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MICROBIAL CELL RECOVERY
FROM SOLID MATERIALS
Final Summary Report

JET PROPULSION LABORATORY
CALIFORNIA INSTITUTE OF TECHNOLOGY
PASADENA, CALIFORNIA

MICROBIAL CELL RECOVERY
FROM SOLID MATERIALS
Final Summary Report

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MICROBIAL CELL RECOVERY FROM SOLID MATERIALS

(Mod. 3)

VOLUME I

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I. SUMMARY OF DEVELOPMENT OF RECOVERY METHODS

Microorganisms entrapped within solids constitute unique contamination control problems. Bacterial spores encased in plaster of paris, asbestos plastic cement, various plastic potting materials, and solid rocket propellants are more resistant to dry heat than those residing on surfaces (ref. 1). Due to this increased resistance to the effects of dry heat it was deemed necessary to develop assay procedures which would allow the detection of very small populations of microorganisms within the solids. Some solids within the spacecraft may be subjected to temperatures in excess of sterilization requirements and thus constitute a minimum contamination control problem. There are, however, solid components which are adversely affected by such treatment. It is essential that the microbial population within these solid materials be accurately known in order to assess their contribution to the total contamination load of the entire spacecraft.

Not only is it essential to develop adequate pulverization techniques for exposing these micro-organisms, but it is also necessary to develop methodology for overcoming the adverse environments provided by the spacecraft solids. At the time of fabrication of spacecraft components, microorganisms from the environment may exist and be incorporated as spores or as vegetative cells in any of a wide variety of physiological states. Obviously, the effects of various plastics, propellants, and other solids on the physiological states of microorganisms are important in the development of suitable recovery techniques. The ultimate aim of pulverization is to reduce the particle size to that which will statistically provide for optimum diffusion of nutrients into the microorganisms and thereby permit the outgrowth of these cells from their encased sites within the solid particle. Since bacterial and fungal spores are best adapted for survival in the internal regions of solids, the pulverization process must be optimized for their detection and cultural recovery. In the course of this pulverization, large microorganisms including fungal spores, mycelia, and large vegetative cells may be physically damaged. Thus, in order to assure recovery of such large cells, additional samples of each of the solids should

be pulverized in a manner modified for the recovery of large cells.

A wide range of substances are present within solids which interfere with the growth of microorganisms. Some plastic solids are polymerized through the use of free radical initiators. These initiators, or their products, may be present in minute but detectable quantities in some solids and must be neutralized in order to provide optimal recovery conditions. Solid rocket propellant contains large quantities of inhibitory substances, which must be leached from the pulverized propellant and neutralized prior to the application of culture techniques. Epoxy resins, coating materials, potting compounds and adhesives contain residual epoxide groups. Such functional groups must be reacted to prevent their interference with recovery of microorganisms.

II. INTRODUCTION

This final report on SN-42 (JPL Contract No. 950740) constitutes the pertinent data obtained in our investigations on the development of methods for the recovery of microorganisms from the interior of solid materials. In order to provide a high degree of reliability of microbial decontamination, it is essential that methods for the detection of viable microorganisms be developed and that these be as sensitive and reliable as possible. In the first phase of this program, under the direction of Dr. John Opfell, the investigation of a variety of methods for detecting the presence of viable microorganisms within solid materials were studied. These methods included culturing, electron spin resonance, electrophoresis, nonfluorescent staining, fluorescent staining, and autoradiography. As a result of these studies conducted in the application of the above methods, it became clear that with the exception of rather limited usefulness of fluorescent staining techniques, culturing represents the most useful, sensitive and easily applied method.

Electron spin resonance spectrometry involves the absorption of incident energy (usually in the microwave region) by unpaired electrons under the influence of a magnetic field. Electron spin resonance measures the magnetic moments of unpaired electrons and are somewhat analogous to the vibrational-rotational transitions in other forms of spectroscopy. Because free radicals occur in the process of metabolic activities within cells, this technique was studied to determine its potential usefulness as an independent means of assessing the presence of microbial contamination in solid materials. Indeed, it was found that differences existed between viable and nonviable microbial cells. Unfortunately, the order of magnitude of such differences is not sufficient to differentiate between other free radicals which may occur in a variety of nonliving solid materials (both organic and inorganic) of a wide variety of types. Since free radicals cannot be distinguished from those generated by small numbers of living microorganisms and inanimate solid particulates, this method was not considered to be of sufficient usefulness to warrant further investigation.

The cell walls of microorganisms contain chemical functional groups which vary in the nature and distribution of electrical charges. A wide variety of

microbial stains adhere strongly to the cell walls, and in some cases to the cytoplasmic constituents of microbial cells. The specificity and the degree of such staining is dependent upon the nature and distribution of the charge groups within the cell wall and the cytoplasm. In the application of this tool for the detection of microorganisms in solids, it is essential that the microorganism exposed from the solid be easily differentiated by microscopy from the debris. Not only is this of importance to determine the presence of microorganisms, but it is of greater usefulness to determine whether these microorganisms are viable or nonviable. Spores of B. subtilis var. niger, Clostridium sporogenes, and Ulocladium were obtained and the viability assessed by cultural methods. An aliquot of each of these microorganisms was subjected to the killing effects of dry heat, autoclaving, exposure to ethylene oxide, formaldehyde, and chlorine gas. Subsequently, both the viable and the nonviable organisms were stained and examined microscopically. No differences were noted that would permit the differentiation between viable and nonviable microorganisms. This method was abandoned.

A number of fluorescent dyes bind strongly to microorganisms. When these microorganisms are examined under a microscope equipped with suitable optics, the background may be adjusted to a nearly uniform black while the outlines of the microorganisms present a bright fluorescence. Thus, it would appear that this technique would be more useful than the nonfluorescent staining techniques since there are fewer interfering and confusing structures in the background. This method is only useful when the solid material and the other debris from the pulverized solid did not possess chemically charged groups which bind the dye. In the latter case the solid as well as the microorganism would show fluorescence and the method would be useless. Likewise it is necessary that the solid not be fluorescent. Under the experimental conditions of comparing viable and nonviable microorganisms, it was not possible to correlate the degree of fluorescence with viability. Thus this method suffers a severe deficiency. In addition, it is an exceedingly slow and painstaking technique which requires a high degree of standardization in order to use for the detection of microorganisms. The fundamental reason for abandoning this method was the lack of reliability in distinguishing viable cells from nonviable cells.

← This material would make a good case for culturing only as proof of viability - if it is indeed ever needed.

The incorporation of radioactively tagged beta emitting isotopes provide a means of determining potential cellular viability. The presence of the tagged metabolite may then be determined by the use of radioautography. The latter technique utilizes a sensitive fine grained photographic emulsion which is sensitive to weak beta emitting radiation. These weak beta rays interact with the photographic emulsion to provide opaque silver grains in a manner somewhat analogous to the effect of light on photographic emulsion. The presence of grain patterns which are confined within the outlines of the microbial cells, or fragments thereof, would provide evidence of viability, assuming that adequate controls are included. When the opaque silver grained pattern develops in regions outside the cell wall area, it may be possibly due to chemabsorption of the radioactive metabolite by the solid or inadequate washing. The resolution of this method is highest when the weakest forms of radiation capable of activating the silver particles in the emulsion is utilized. Maximum resolution depends upon a minimum distance between the microorganism containing the radioactive metabolite and the photographic emulsion. When spores of B. subtilis var. niger were incubated with solutions containing tritium labeled thymidine no radioactivity was detected in the radioautographs. This was because DNA is only duplicated during cell division which does not take place within spores.

Culturing represents the most reliable method thus far devised for the detection of viable microorganisms. This method depends on the ability of the microorganisms to undergo cellular division. There are, of course, deficiencies in the use of this method since physical and/or chemical trauma associated with the exposure of the microorganism from its location within the solid may result in a lack of ability to demonstrate the organisms' viability by culture methods. Thus, culture methods have long been established as useful techniques for the detection of viable microorganisms, and therefore, were used in Phases 2, 3, and 4.

Not only is it essential to develop adequate pulverization techniques for exposing these microorganisms, but it is also necessary to develop methodology for overcoming the adverse environments provided by the spacecraft solids. At the time of fabrication of spacecraft components, microorganisms from the environment may exist and be incorporated as spores or as vegetative cells in any of a wide variety of physiological states. Obviously, the effects of various plastics, propellants, and other solids on the physiological states of microorganisms

are important in the development of suitable recovery techniques. The ultimate aim of pulverization is to reduce the particle size to that which will statistically provide for optimum diffusion of nutrients into the microorganisms and thereby permit the outgrowth of these cells from their encased sites within the solid particle. Since bacterial and fungal spores are best adapted for survival in the internal regions of solids, the pulverization process must be optimized for their detection and cultural recovery. In the course of this pulverization, large microorganisms including fungal spores, mycelia, and large vegetative cells may be physically damaged. Thus, in order to assure recovery of such large cells, additional samples of each of the solids should be pulverized in a manner modified for the recovery of large cells.

A wide range of substances are present within solids which interfere with the growth of microorganisms. Some plastic solids are polymerized through the use of free radical initiators. These initiators, or their products, may be present in minute but detectable quantities in some solids and must be neutralized in order to provide optimal recovery conditions. Solid rocket propellant contains large quantities of inhibitory substances which must be leached from the pulverized propellant and neutralized prior to the application of culture techniques. Epoxy resins, coating materials, potting compounds and adhesives contain residual epoxide groups. Such functional groups must be reacted to prevent their interference with recovery of microorganisms.

III. METHODS AND RESULTS

A. Pulverization

The pulverization of solid materials used in the fabrication of spacecraft may be accomplished through the use of high speed blenders, grills, mortar and pestle, ball and vortex mills, abrasive devices, and saws. (See references 2 to 9). These methods, each have their characteristic advantages and disadvantages. Control of the extent and uniformity of pulverization with these methods is difficult and in some cases not possible. The production of aerosols limits the usefulness of the high speed blenders and drilling techniques. With certain types of solids, explosions occur when the level of pulverization reaches a certain particle distribution range. The recovery of micro-organisms pulverized in solids using high speed blenders and drills was disappointingly low. It would appear likely that the lack of adequate control of heat generated in these forms of pulverization as well as the physical trauma incident to these methods may be responsible for such results. Sawing permits the control of both the heat production (by controlling the sawing rate) and the extent of pulverization through selection of the ideal tooth design.

Various blade and tooth confirmations produce widely different forms of pulverization. The types of saw blades used in the sawing of the various types of materials are numerous; however, the major parameters of importance in determining the types of pulverization produced are dependent upon the following factors; 1) tooth confirmation and design, 2) pitch (number) and size of teeth, 3) sawing rate, 4) pressure applied to the solid.

The most important factor affecting the effectiveness of pulverization using saw blades can be related to the design and configuration of the teeth. The angles of the tooth face, back, clearance and rake, in addition to the tooth spacing and go up depth are illustrated in Figure 1. There are several possible combinations of teeth configurations which produce widely different results in the pulverization of solids. The short nearly vertical angle tooth design typical of hacksaw blades appears to be the most useful for the

pulverization of rigid plastic solids.

There are a variety of ways in which the teeth are set (Figure 2). The alternate set teeth have one tooth to the right and the next to the left. This type of set is common for some types of plastic cutting blades and for nearly all wood cutting blades. The configuration has not proven to be effective in the pulverization of solids, since it produces large cuboidal chips. By adding one straight tooth between the pairs of right and left teeth, the type of chips that are produced differ. If the angle of the cutting blade is acute in respect to the surface of the solid, a ribbon is produced in place of a chip.

The raker set, which interposes a straight tooth between the left and right set of teeth with the hacksaw configuration, is capable of clearing the ribbon and partially disrupting it. The most common blade, containing some 22 to 32 teeth per inch, employs wavy set teeth. These teeth are formed by alternately bringing two or more teeth to the right and to the left in graduated degrees. In this configuration, a center line drawn through the center of each tooth would generate a smooth low pitch wavy curve. The blades with 32 teeth per inch were generally gang set. In this configuration the tooth edge portion of the blade was formed with a device which gang sets 6 to 8 teeth in one direction and alternates the set of the succeeding group of 8 teeth so as to form a wavy pattern throughout the entire length of the blade. These blades are designed for cutting. Among the commercially available blades, the fine tooth blades show advantages over the alternate or raker type configuration in pulverization of solids.

In spite of the fact that hacksaw blades are designed for cutting hard metals, it is desirable to use nickel molybdenum alloy blades or tungsten steel blades in order to minimize the rate of dulling on the blade. Dulling which occurs when using other types of hardened steel alloys may result in poor pulverization. Cross linked epoxy polymers with fillers cause surprisingly rapid dulling of blades. The types of blades studied

included a representative range of metal alloys.

The pressure applied to the blade during the sawing processes is of importance. Ideally this pressure should not exceed 100 grams per inch. The greater the amount of weight on the blades, the thicker the resulting ribbon. The length of blade is of significance since it is convenient to use slow long strokes which utilize the entire length of the blade and thus allow a more even continuous stroke and increase the uniformity of pulverization. The effective pressure vs. blade design for the pulverization of plastic materials studied is shown in Tables 1a to 1c.

Solid rocket propellant is the most difficult spacecraft component material to pulverize. The Dremel drill, slicing with the microtome and other methods which were studied were ineffective because of the peculiar characteristics in this substance (ref. 4). Sawing with appropriate types of hacksaw blades provided the most efficient pulverization. The particles produced were sticky and tended to agglomerate. However, the individual particles even though agglomerated in the culture media provided sufficient surface area for effective recovery of microorganisms (5). The particles produced by sawing differ depending upon the amount of pressure applied and the rate of sawing. Ribbon-like structures produced when the sawing pressure was less than 100 grams per inch were less than 15 microns in diameter and displayed a high instance of micro cracks and imperfections radiating in all directions, Figure 3. Care must be taken that the particles are collected and handled in such a way that agglomeration of particles is minimized. The typical microscopic appearance of this material is shown in Figures 4 and 5.

Polyester and epoxy resins are satisfactorily pulverized by sawing. Ribbons are produced whose thicknesses are generally 5 to 15 microns when a minimum amount of pressure (less than a 100 grams per inch) is applied by the blade to the plastic. The rigid highly cross-linked or filled rigid plastic tend to pulverize as a powder. The less rigidly cross-linked plastics and semi-flexible types readily form ribbons (Figures 6 to 9).

If we assume that a microbial spore in an idealized model will occupy a space of one cubic micron, in the absence of trauma, toxicity, and neglecting die away, the solid when pulverized to this size range should provide a theoretical recovery of 100 percent. If we further assume that the model for the solid particle is two microns on each side, the volume is increased eight-fold (Figure 10). Is it reasonable to suppose that the theoretical recovery is decreased by a similar ratio of volume increase. The worst case would require that the spore exist in the center of all planes within the cube. Under these conditions, the maximum distance between the spore and the exterior would be one half micron. The probability of this worst case would approach zero. The physical constraints of a particle containing the entrapped spore would be less than the theoretical cube case within any particle of two microns or less actually observed in the pulverized sample.

The worst case situation in such a particle would require that there be no micro cracks or imperfections in any plane which would encroach upon the central location of the spore. The probability of this occurring also approaches zero. Particles resulting from the pulverization showing a maximum dimension in any plane are generally sized by that dimension. Nearly all of the particles, with the exception in the longest plane, may be a small fraction of 5 microns. Statistically, nearly all such particles would certainly have, in one plane or another, distances much less than 5 microns. In the course of our studies on pulverization, we have observed that well over 80% of such classified particles contain cross-sectional distances of less than 2 microns. It might be noted that in Table Ic, when using a 32 tooth blade and light pressure, as much as 70% of the particles were less than 5 microns in size.

This material proves the need for ^{various} work on recovery methods

B. Leaching and Neutralization

Often soluble toxic substances may be extracted from pulverized solid materials. In some instances, simple dilution of the inhibitory substance is sufficient to allow nearly maximum growth rates in a reasonable final culture volume. Leaching may in some instances be necessary when inhibitors or toxic materials are not neutralizable. An important category of leachable inhibitors are polymerization, cross-linking agents, and catalysts. Solid plastic materials are polymerized by a variety of agents. Examples are benzoyl or isobutryl peroxide, epoxides, formaldehyde, or epichlorhydrin. The neutralization of benzoyl and isobutryl peroxide are summarized in Table 2. Of the various reactants studied, glutathione has proven to be the most effective and the least toxic. The other reductants investigated included sodium borohydride and cysteine.

While all these reductants were effective in the adjustment of the redox potential, it was found that cysteine sulfoxide was formed as a reaction product. This compound was toxic to some microorganisms. In addition, cysteine reacts with SS bonds within proteins. This amino acid can also react with transition state metals and thus interfere with trace metal metabolism of microorganisms when this amino acid is used in moderate to high concentrations. Borohydride is toxic to microorganisms largely due to its reaction product, borate ion. So in spite of its effectiveness in the adjustment of redox potential when used in dilute solutions, its slight toxicity limits its usefulness.

Virtually all epoxides and trimethylene oxides as well as tetramethylene oxides may be used in the synthesis of plastic materials. Tetrahydrofurane will polymerize readily only when cationic catalysts are used. Each of the above compounds show evidence of various degrees of toxicity. The epoxides are most toxic to both vegetative cells and spores. Tetrahydrofurane displays far less toxicity, and in many instances no toxicity at all.

The most important class of plastic materials utilized in potting compounds, electronic components, and encasement materials are the epoxy resins. A wide range of physical characteristics may be obtained with these compounds depending upon the types of monomers utilized and the nature and composition of the hardeners. A typical type of epoxy resin may be prepared by the base induced condensation of a polyhydroxy compound, usually a bisphenol, and utilizing in most cases epichlorhydrin which forms an intermediate of low molecular weight. This compound is essentially a linear polymer, containing terminal epoxide groups and pendant hydroxyls, Figure 11. An excess of epichlorhydrin in the reaction results in the termination of the polymeric chains with epoxy groups. These unreacted epoxide groups cause most of the toxicity associated with epoxy resins. The resins may be reacted to achieve maximum cross-linking by using curing or cross-linking agents, examples of which are amines and carboxylic acids and their anhydrides. In nearly all of the currently marketed epoxy resins epichlorhydrin is used.

Among the various methods of neutralizing this toxicity which were devised in our laboratory are 1) adjustment of pH to a moderately acidic concentration (pH 3 to 4) to open the epoxide ring or 2) reacting the epoxide with a suitable compound to form a chemical derivative. Both of these approaches were investigated and the results are summarized in Table 3. The reaction of epoxides with proteins is complex and must require some elaboration. It was discovered that this interaction at physiological pH's involved chiefly the amino acid histidine.

Epichlorhydrin was allowed to react with 2% casein hydrolysate for a period of 4 hours at 25°C. The amino acid which reacted most vigorously at pH 7.0 was histidine. The amino groups of some of the other amino acids were also reactive. The extent of reaction of the various amino acids in the mixture are summarized in Table 4. Epoxides may react with nucleic acids. The heterocyclic portion of the molecule may also react; however, it is of less importance. Epichlorhydrin exerts its more profound biological effects on DNA by causing either intramolecular cross-linking, cross-linking with

neighboring functional groups within the molecule, or with the nucleohistone present in the vicinity of the DNA or intramolecular cross-linking. The various forms of interaction are summarized in Figure 12. Nucleic acids were active in the neutralization of epichlorhydrin.

Beef extract (Difco Corporation) and RNA at 5% concentrations were effective neutralizers for epichlorhydrin. These substances form a viscous suspension at 5% concentrations. Higher concentrations of these neutralizers result in markedly inhomogeneous suspensions and do not improve the effectiveness of the neutralization (Table 5).

Leaching procedures were applied to Eccocoat 1C2 in an effort to improve the recovery from this solid (Table 6). The most promising leach fluids were ethylene chloride, chloroform, and trichlorotrifluoroethane. Slightly higher recoveries were obtained at 5°C than at 25°C. Since the order of magnitude of recovery remained low with the leaching procedures attempted, we abandoned leaching methods for Eccocoat 1C2 and Epon 901. Recoveries were sufficiently high for Stycast, Paraplast, Parlodion and Maraset compared to other data (4) not to apply leaching techniques. Leaching is used together with neutralization for solid rocket propellant (5).

Ultrasound at maximum output was useful in increasing the recovery from Stycast 1090 (Table 7).

C. Recovery of Microorganisms from Solids

All of the test organism types were recovered from Paraplast (Table 8). The vegetative cells were recovered from the latter solid only. Lower recoveries were obtained from Eccocoat 1C2, Epon 901, and RTV 40. Methods used were outlined in the protocol.

D. Culture Media

A variety of culture media including Bacto Anaerobic Agar*, Bacto Blood Agar Base, Thioglycollate*, Trypticase Soy**, were studied during the course of the past two years for the recovery of microorganisms from solids. The medium shown on Table 9 has proven to be the most effective. Although the termination of

*Difco Laboratories, Detroit, Michigan

** Baltimore Biological Laboratories

dormancy and the initiation of germination do not require special steps with the spores specified in this study, ingredients were incorporated in the growth medium to optimize these phenomenon, (tetrahydrofurane, alanine and casamino acids).

IV. FINAL PROTOCOL FOR THE CULTURING OF MICROORGANISMS FROM THE INTERIOR OF SOLID MATERIALS

A. Assembly of Sterile Chamber

A rectangular container (width 50 cm, length 100 cm having a capacity of 75 liters) is prepared utilizing 4-8 mil polyethylene tubing. A gland is provided which allows for exhaustion of the ethylene oxide and subsequent refilling with air. An aluminum frame and base supports the structure and provides a surface for the attachment of a vise. The contents of the chamber include ethylene oxide, the hacksaw assembly, liquid media, the sterile solutions of neutralizers, sterile standardized acid and base, and the solid materials to be studied. Several additional culture vessels are included which are utilized as controls by inference to demonstrate that the environment inside of the bag is sterile (Table 10).

Following the assembly of the contents of the bag, it is sealed utilizing a double heat seal seam. The bag is tested to determine whether leaks are present using a thermal conductivity cell of the gas chromatograph as a detector of leakage.

B. Surface Decontamination with Sterilant Gas

Evaporation of a previously determined volume of ethylene oxide in sufficient excess to maintain a concentration of 500 mg/L of space is allowed to take place within the bag at room temperature for a period of 12-16 hours. The sterilant gas mixture employed consists of 12% ethylene oxide and 88% Freon-12 or pure ethylene oxide.

Six test tubes each containing 10^2 spores of B. subtilis var niger are used as controls on the effectiveness of the surface decontamination procedure. These tubes are capped in a manner which permits the gas phase to enter. At the end of the surface decontamination period, 10 ml of growth medium is added and the tubes still inside the bag are incubated under the same conditions as the assay tubes. The screw capped tubes containing the culture media and the neutralizer are sealed to prevent contact with the ethylene oxide. No evidence of outgrowth has been obtained in those tubes in the tests reported in this study.

C. Analysis of Ethylene Oxide Concentration

Gas samples for the assay of ethylene oxide and humidity were collected using a 10 ml syringe. The needle is introduced into the bag at the site of a double rubber seal. Past experience has shown that leakage from this sampling site does not take place; however, added assurance of protection against this is provided by using pressure sensitive tape over the puncture area. Ethylene oxide escapes from the bag at a very slow rate thus necessitating an additional ethylene oxide analysis at the end of the decontamination period. The humidity and ethylene oxide concentrations are determined by gas chromatography utilizing the same gas chromatographic column (1% carbowax 1540 on Teflon 6) using a gas chromatograph equipped with a thermal conductivity detector (Carle Instruments, Inc.). This instrument can detect 10^{-6} g of ethylene oxide or H_2O/ml at maximum sensitivity.

D. ~~Exhaustion~~ Exhaustion of Ethylene Oxide and Replacement with Air

Following the sterilization cycle, the residual ethylene oxide is exhausted from the bag, and the chamber is flushed 5 times with alternate filling and exhausting sterile air. This is facilitated by using a tube packed with sterile bacteriological cotton which serves to remove gross particles and microorganisms passing through the tubing line from the exterior of the bag. Also in this line, a Millipore filter provides an additional barrier to the contamination of the bag through this line. To further insure that no remaining traces of ethylene oxide are present within the bag, a 10 ml volume of the air within the bag is withdrawn and injected into the gas chromatograph to determine the concentration of ethylene oxide.

E. Inoculation and Pulverization of the Solid

The plastic and hardener are weighed out and mixed according to the manufacturer's directions. The mixing is accomplished under clean conditions. The inoculation of the solid is performed just prior to its solidification. Normally, at this stage the solid has warmed appreciably

due to the exothermic reaction taking place. If the temperature is in excess of 35°C, it is cooled to or below that temperature prior to the addition of the inoculant. After the inoculant is added, it is quickly stirred and tamped in order to release any gas bubbles present within it. After the solid has solidified, it is removed from the mold and subjected to pulverization procedures.

The solid is placed in the vise, the collector is placed beneath the solid and the sample sawing is performed. The pressure of the blade on the solid is of critical importance since it determines to a major extent the thickness of the spiral-shaped ribbons that are formed. The weight of the holder and blade/inch of blade constitutes the only pressure applied during the sawing process. Under ideal circumstances, the pressure should be 100 gm/inch or less. The rate of sawing determines the amount of heat produced at the interface of the blade and the solid (5). The weight of the sample is estimated using a 5 ml beaker. This does not provide sufficient accuracy but allows crude estimations of the quantity of the solid processed. (The exact weight of solid added may be determined at later steps when the bottle containing the solid is subjected to culturing. The tared weight of the bottle and estimation of all subsequent volume permits the estimation of the total weight of the solid sample).

In spite of the fact that hacksaw blades are constructed from high-grade hard steel alloys, they dull at surprisingly fast rates. With epoxy solids, the blade should be changed after the pulverization of 5 grams of solid. The changes observed when pulverization is accomplished with a dull blade are characterized by ribbons of irregular thickness and a tendency of the solid to break into large chips.

Current work indicates that it is easier to disintegrate the solid material if it is subjected to a brief grinding with neutralizer in a mortar and pestle. This grinding is carried out with a pestle chilled by placing a corner of the bag containing the pestle in an ice bath. Exposure of the contents of the mortar and pestle to ultrasound, utilizing a Branson S-75 ultrasound stephorm at an output of 5 amps for thirty seconds, increases the recovery of spores. The bag decreases the efficiency of the ultrasound probe and thus it is necessary to use full output.

F. Neutralization

It is convenient to add the neutralizer immediately following the sawing step (5% RNA, 5% beef extract). The quantity of the neutralizer used in these experiments is 2 ml per gram of solid.

At the end of the grinding and ultrasound period, the sterile culture medium maintained at 40°C by a water bath is added to additional neutralizer (8 ml). The mortar and pestle are withdrawn from the ice bath and allowed to sit for one hour to allow the neutralizers to overcome the growth inhibitory substances present. The contents of the mortar following sufficient mixing to produce a homogeneous mixture are added to a test tube and the test tube closure is sealed. Care must be taken in the latter steps to accomplish the mixing with sufficient rapidity so that the semisolid agar medium-neutralizer mixture does not solidify prematurely.

The adjustment of pH, redox potential, and control of fluidity are performed within the sterilant bag. Model experiments previously performed outside of the bag using the same weight of sample provide the volume requirements for the oxidants, reductants and other such additives. These manipulations prevent contamination by pH meters, volumetric burets and such instrumentation if they were included within the bag.

G. Leaching

Leaching the pulverized solid at 5°C is partially effective in removing growth inhibitors of certain solids. The use of neutralizers as outlined above is generally as effective in overcoming the toxicity of the solid. The use of both neutralizers and leaching steps does not at this time appear to be warranted since the increase in recovery when both procedures are applied is not sufficiently high to be useful.

H. Culturing

The composition of the culture media used in these experiments is shown in Table 9. This growth medium was devised to permit the growth of a wide variety of spore forming microorganisms in addition to B. subtilis.

X

The culture tubes with the closures tightly in place are incubated at 32°C. With B. subtilis var niger, the maximum number of colonies is observed following an incubation period of three to four weeks.

Nondamaged spores incubated in this culture medium grow out rapidly with maximum number of organisms evident within a period of one to two days. It is suggested that the delayed appearance of colonies is dependent upon chemical and/or physical injury sustained in the incorporation into the solid epoxy resin or in the process of recovery from the resin.

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TABLE I-A

SIZE OF PARTICLES AND INTERVALS BETWEEN CRACKS AND IMPERFECTIONS

(STAR BLADES No. 1218-3 (12 x 1/2 x .025 - 18 teeth)

Plastic	Vertical Pressure (grams) Sawing	% of Particles (Average of 3 Replicates)									
		Interval between cracks and size of particles (microns)									
		<1	1	1-2	2-5	5-10	10-20	20-40	>50		
Maraset	19	4	9	11	20	15	26	8	7		
	152	2	4	8	22	19	26	10	9		
	546	2	3	4	9	13	27	18	24		
Stycast	19	5	8	10	19	16	28	9	5		
	152	3	5	9	20	22	25	9	7		
	546	1	5	7	11	19	30	19	8		
Epocast 4D	19	2	5	12	21	17	25	12	6		
	152	1	4	7	24	21	26	11	6		
	546	2	2	4	13	23	27	20	9		
Epocast 4H	19	3	7	11	21	15	28	11	4		
	152	1	5	7	21	20	27	10	9		
	546	2	4	6	12	22	29	17	10		
Epocast 4B	19	1	7	9	23	19	27	11	3		
	152	2	5	8	25	17	28	6	9		
	546	1	2	5	13	25	28	18	8		

SIZE OF PARTICLES AND INTERVALS BETWEEN CRACKS AND IMPERFECTIONS

(STAR BLADES NO. 1218-3 (12 x 1/2 x .025 - 24 teeth))

Plastic	Vertical Pressure (grams) Sawings	% of Particles (Average of 3 Replicates)									
		Intervals Between Cracks and Size of Particles (microns)									
		<1	1	1-2	2-5	5-10	10-20	20-40	>50		
Maraset	19	17	23	17	11	12	12	4	4		
	152	9	14	19	21	17	10	7	3		
	546	3	6	10	14	21	12	14	20		
Stycast	19	11	13	16	12	19	19	7	3		
	152	6	8	9	20	18	20	11	8		
	546	3	8	10	16	20	24	11	7		
Epocast 4D	19	13	21	18	11	13	9	8	7		
	152	10	21	12	15	12	17	6	7		
	546	5	11	15	14	17	23	9	6		
Epocast 4H	19	9	14	17	10	16	12	7	5		
	152	8	10	11	19	17	19	10	6		
	546	4	9	12	15	19	23	11	7		
Epocast 4B	19	12	22	16	10	16	11	8	5		
	152	7	14	11	21	17	18	7	5		
	546	4	10	14	14	19	22	10	7		

SIZE OF PARTICLES AND INTERVALS BETWEEN CRACKS AND IMPERFECTIONS

(DISTON BLADE SPECIAL ALLOY DURAFLEX NO. F-1232 (12 x 1/2 x .025 - 32 teeth))

Plastic	Vertical Pressure (Grams Sawing)	% of Particles (Average of 3 Replicates) Intervals Between Cracks, Size of Particles (microns)								
		<1	1	1-2	2-5	5-10	10-20	20-40	>50	
Maraset	19	21	26	16	12	6	10	4	4	
	152	11	17	21	23	15	6	5	2	
	546	2	7	12	15	20	10	12	22	
Stycast	19	13	15	17	14	19	11	5	6	
	152	7	10	9	19	18	16	13	8	
	546	5	9	12	17	21	17	7	12	
Epocast 4D	19	19	25	20	12	7	8	3	6	
	152	16	26	19	13	11	10	5	9	
	546	7	13	23	17	12	12	6	10	
Epocast 4H	19	17	20	19	11	15	9	4	5	
	152	9	17	15	13	19	15	5	7	
	546	5	9	12	9	25	18	10	12	
Epocast 4B	19	13	19	19	18	16	6	6	3	
	152	9	20	16	12	22	9	7	5	
	546	8	12	14	13	19	14	9	11	

TABLE 2-A

NEUTRALIZATION OF BENZOYL AND ISOBUTYRYL PEROXIDES

<u>Compound</u>	Percent of Control Counts (Average of 5 plate counts)			
	Sodium Borohydride 0.1M	Cysteine 0.1M	Glutathione 0.1M	Water
Benzoyl Peroxide, 5%	73	52	92	0
Isobutyryl Peroxide, 5%	65	50	88	0
Control (H ₂ O)	100	100	100	100

After reduction of the peroxide, 10^3 spores of B. subtilis var niger were added. After exposure for 2 hours at 25°C, the organisms were counted by plate dilution technique subsequent to incubation at 35°C for 24 hours.

TABLE 2-B

NEUTRALIZATION OF BENZOYL AND ISOBUTYRYL PEROXIDES

<u>Compound</u>	Percent of Control Plates			
	Sodium Borohydride 0.1M	Cysteine 0.1M	Glutathione 0.1M	Water
Benzoyl Peroxide, 5%	12	18	27	0
Isobutyryl Peroxide, 5%	17	20	32	0
Control (H ₂ O)	89	99	100	100

After reduction of the peroxide, 10^3 spores of C1 sporogenes were added. After exposure for 2 hours at 25°C, the organisms were counted by plate dilution technique subsequent to incubation at 35°C for 24 hours.

TABLE 2-C

NEUTRALIZATION OF BENZOYL AND ISOBUTYRYL PEROXIDES

<u>Compound</u>	Percent of Control Plates			
	Sodium Borohydride 0.1M	Cysteine 0.1M	Glutathione 0.1M	Water
Benzoyl Peroxide, 5%	2	3	14	0
Isobutyryl Peroxide, 5%	6	8	16	0
Control (H ₂ O)	50	64	97	100

After reduction of the peroxide, 10^3 cells of E. coli were added. After exposure for 2 hours at 25°C, the organisms were counted by plate dilution technique subsequent to incubation at 35°C for 24 hours.

TABLE 2-D

NEUTRALIZATION OF BENZOYL AND ISOBUTYRYL PEROXIDES

<u>Compounds.</u>	Percent of Control Plates			
	Sodium Borohydride 0.1M	Cysteine 0.1M	Glutathione 0.1M	Water
Benzoyl Peroxide, 5%	47	51	66	1
Isobutyryl Peroxide, 5%	40	47	52	2
Control (H ₂ O)	82	98	100	100

After reduction of the peroxide, 10^3 spores of Ulocladium were added. After exposure for 2 hours at 25°C, the organisms were counted by plate dilution technique subsequent to incubation at 35°C for 24 hours.

TABLE 3

NEUTRALIZATION OF EPICHLORHYDRIN

% of Control (Average of 3 Replicates)

Treatment	<i>B. subtilis</i>	<i>C. sporogenes</i>	<i>S. marcescens</i>	<i>Ulocladium</i>	<i>M. epidermitidis</i>	<i>E. coli</i>
5 ml H ₂ O	0	0	0	0	0	0
Adjust pH to 3, hold 2 hours, readjust to 7	42	32	12	0	0	0
Adjust pH to 4, hold 4 hours, readjust to 7	28	11	7	0	0	0
Adjust pH to 4, hold 4 hours, presence of 5% histidine, readjust to 7	67	50	20	0	0	0
Adjust pH to 4, hold 15 minutes, readjust to 7	12	7	4	0	0	0

10⁴ organisms were added to a final volume of 3 ml of 5% epichlorhydrin immediately treated as indicated, and brought to a final volume of 5 ml. Recovery of viable organisms was measured as percent of control unexposed to epichlorhydrin. The organisms were counted by plate dilution technique subsequent to incubation at 35°C for 48 hours.

TABLE 4

THE EFFECT OF EPICHLORHYDRIN ON THE AMINO ACIDS OF CASEIN

<u>Amino Acid</u>	<u>AMINO ACID COMPOSITION (%) (Average of 3 Replicates)</u>	
	<u>Before Reaction</u>	<u>After Reaction</u>
Arginine	4.3	4.1
Histidine	2.2	0
Lysine	7.5	7.1
Tyrosine	6.9	6.3
Tryptophane	1.2	0
Phenylalanine	4.8	4.2
Cystine	0.35	0
Methionine	3.4	3.1
Threonine	3.8	2.1
Serine	7.6	5.2
Leucine	9.8	8.8
Isoleucine	6.2	5.7
Valine	6.6	6.2
Glutamic acid	23.3	21.3
Aspartic acid	6.0	5.8
Glycine	0.5	0.2
Alanine	5.5	4.1
Proline	7.5	6.2
Hydroxyproline	0.2	0

Two grams of casein were reacted with 100 ml of 0.5% epichlorhydrin at 20°C for 30 minutes at PH 7.0. Note that all of the histidine was destroyed while very little reaction occurred with the other amino acids.

TABLE 5

THE EFFECT OF VARIOUS NEUTRALIZERS ON THE RECOVERY OF MICROORGANISMS

<u>Neutralizer</u>	<u>% of Control (Average of 5 Replicates)</u>		
	<u>B. subtilis</u>	<u>Cl. sporogenes</u>	<u>S. marcescens</u>
Histidine, 5%	12	8	10
Casein, 5%	3	0	0
Beef Extract, 5%	25	17	21
Beef Extract, 10%	28	24	25
RNA, 5%	22	20	22
RNA, 10%	20	-	19
RNA, 5% and			
Beef Extract, 5%	71	55	61
* RNA 10%, Beef Extract 10%			
Water	0	0	0

10^4 organisms were added to a final volume of 3 ml of epichlorhydrin immediately treated with the neutralizer at 0-5°C in a final volume of 6.0 ml. Recovery of viable organisms was measured as percent of control unexposed to epichlorhydrin. The organisms were counted by plate dilution technique after incubation at 35°C for 48 hours.

TABLE 6

THE EFFECT OF LEACHING ON THE RECOVERY OF ORGANISMS FROM ECCOCOAT 1C2

<u>Leaching Medium</u>	% Recovery (Average of 3 Replicates)							
	<u>B. subtilis</u>		<u>Cl. sporogenes</u>		<u>S. marcescens</u>		<u>Ulocladium</u>	
	5°C	25°C	5°C	25°C	5°C	25°C	5°C	25°C
Water, pH 2	0	0	0	0	0	0	0	0
Water, pH 5	0	0	0	0	0	0	0	0
Water, pH 7	0	0	0	0	0	0	0	0
Ethylene Chloride	3	1	5	2	4	3	0	0
Chloroform	4	1	6	2	5	2	0	0
Acetone	2	0	0	1	1	1	0	0
Trichloro Trifluoro Ethane	5	1	2	0	6	0	0	0
Carbon Tetrachloride	2	0	0	0	0	0	0	0
Acetone: H ₂ O (50:50 vol/vol)	2	2	0	1	2	0	0	0

The organisms were inoculated at a level of 10^3 /gm of solid. The leach fluid 10 ml/gm solid were added to the pulverized Eccocoat, allowed to leach for 2 hours, filtered and cultured on membrane filters. The membrane filter cultures did not show growth and therefore, no values for the leach fluid are shown. The pulverized, leached solid was also cultured and the values reported above. The methods outlined in the protocol section were used for cultural recovery. The cultures were incubated at 35°C. The colonies were counted at 2 week intervals. The counts for these cultures were maximal at 5 weeks of incubation.

TABLE 7
EFFECT OF ULTRASONIC OSCILLATION ON RECOVERY
OF B. SUBTILIS VAR NIGER FROM STYCAST 1090

<u>Time of Exposure</u>	(Average of 3 Replicates) number recovered/gram (247)	<u>% Recovery</u>
0		100
15 secs.		99
30 secs.		118
1 min.		135
2 mins.		144
6 mins.		165
8 mins.		161
10 mins.		169
15 mins.		160
30 mins.		141

TABLE 8

RECOVERY OF MICROORGANISMS FROM SOLIDS

<u>Solid</u>	% Recovery (Average of 3 Replicates)					
	<u>B. subtilis</u>	<u>Cl. sporogenes</u>	<u>S. marcescens</u>	<u>Ulocladium</u>	<u>M. epidermitidis</u>	<u>E. coli</u>
Eccocoat 1C2	2	4	2	0	0	0
Epon 901	4	4	1	0	0	0
Paraplast	78	70	66	29	16	9
Parlodion	51	66	46	3	0	0
Stycast	32	11	19	0	0	0
Maraset	38	5	22	0	0	0
RTV 40	3	1	5	2	0	0

The organisms were inoculated at a level of 10^3 /gm of solid. The methods outlined in the protocol were used for the cultural recovery. Cl. sporogenes was incubated under anaerobic conditions. The colonies were counted at 2 wk. intervals. The counts for these cultures was maximal at 5 weeks incubation.

TABLE 9
CULTURE MEDIUM

	<u>Per cent Composition</u>
Tryptone	5.0
Casamino acids	0.25
Bacto soytone	0.2
Soluble starch	0.5
Dextrose	1.5
d l - Alanine	0.5
DNA	0.2
Tetrahydrofurane	0.5
Yeast extract	0.9
Magnesium sulfate	0.2
Azolectin	0.25
*Tween 80	0.15
Potassium phosphate dibasic	0.5
Agar	0.8
*Polyoxyethylene sorbitan monooleate	

TABLE 10

LIST OF MATERIALS OF ENCLOSURE AND CONTENTS

1. Ethylene oxide or ethylene oxide-Freon 12 mixture.
2. 4-8 mil polyethylene tubing 50-100 cm width of 50-100 cm length.
3. Screw capped culture tubes, 25 cc capacity
 - a. empty
 - b. containing 10 ml of neutralizer (1 for each solid sample of 1 g).
 - c. containing 10 ml culture media (4 for each solid sample of 1 g).
 - d. control tubes previously inoculated with 10^2 spores of B. subtilis var niger
 - e. same as d but inoculated into 10 ml of growth media.
4. Vise mounted on solid surface
5. Saw blade and holder with total weight on sawing edge of 50-100 g/inch
6. Mortar and pestle. Paper wrapped 125-500 ml capacity (1 for each solid to be assayed).
7. Paper towels - 6
8. 45°C water bath
9. Ice bath
10. Screw capped bottles containing
 - a. 50 ml sterile distilled H₂O
 - b. sterile 0.01 N NaOH
 - c. sterile 0.01 N HCl
 - d. sterile 0.1 M glutathione

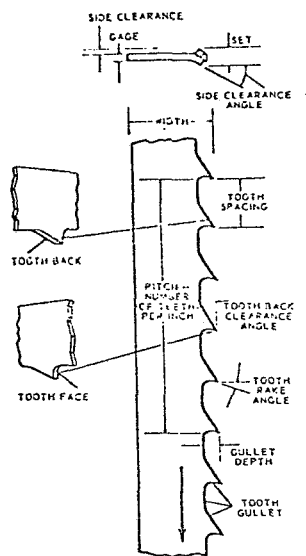


FIGURE 1. The design features of saw blades.

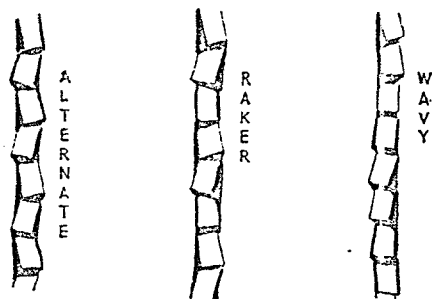


FIGURE 2. Teeth settings of saw blades.

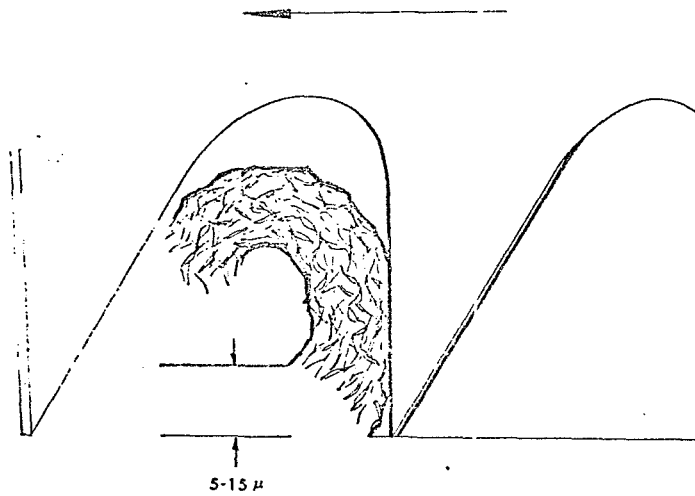


FIGURE 3. Saw - solid interaction.

Large arrow indicates direction of saw movement. In some instances, a ribbon which is highly fractured is produced as shown, whereas, with most brittle solids, fragmentation into small particles occurs.

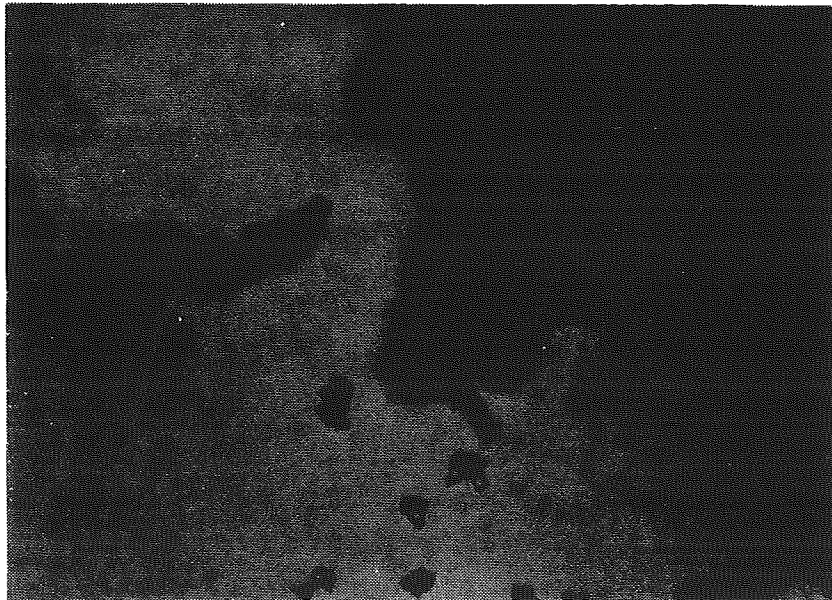


FIGURE 4. Microphotograph of solid rocket propellant.

Note the agglomeration of particles and the presence of fractured ammonium perchlorate crystals.

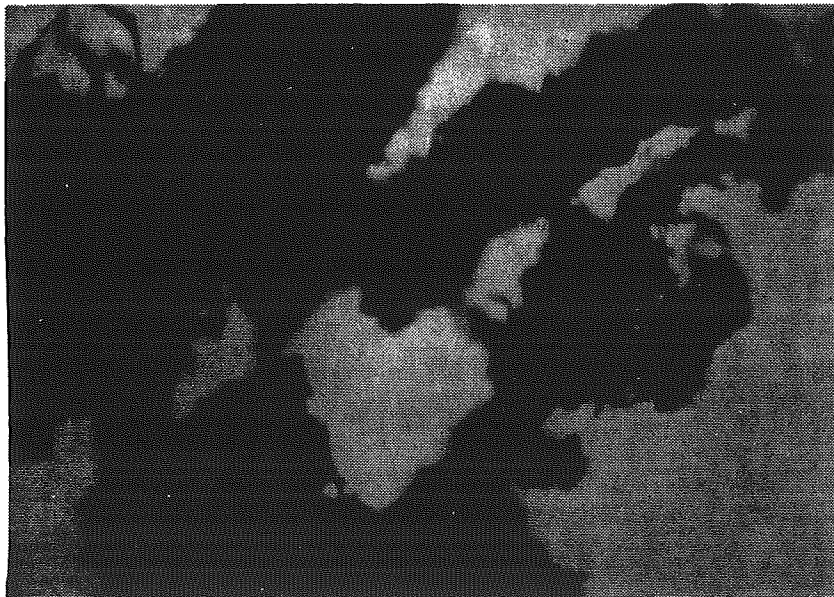


FIGURE 5. Microphotograph of solid rocket propellant in water.

Most crystals of ammonium perchlorate have dissolved,
exposing the binder.

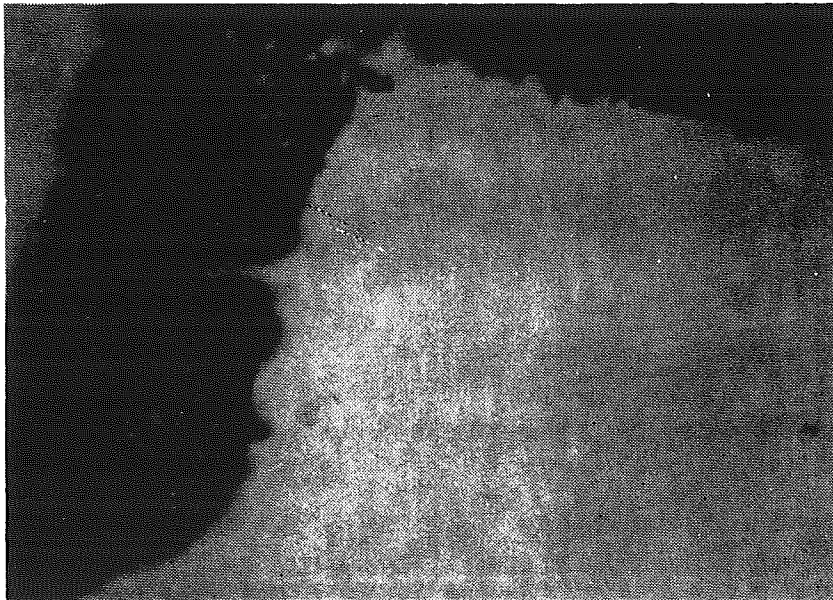


FIGURE 5. Microphotograph of ribbon produced from Epocast 4H.

Note the microcracks, surface irregularities and imperfections within the ribbon.

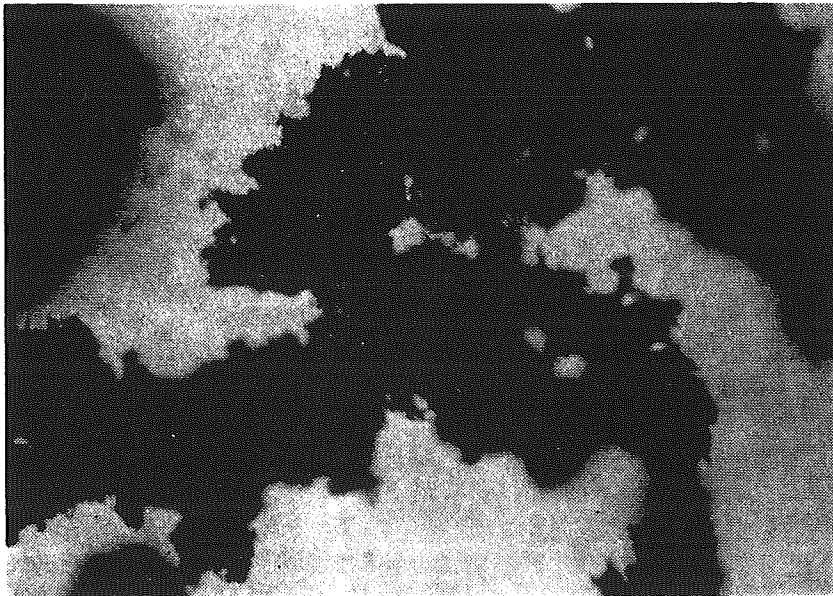


FIGURE 7. Microphotograph of ribbon produced from Epon 901/B1.

Note the microcracks, surface irregularities and imperfections within the ribbon.

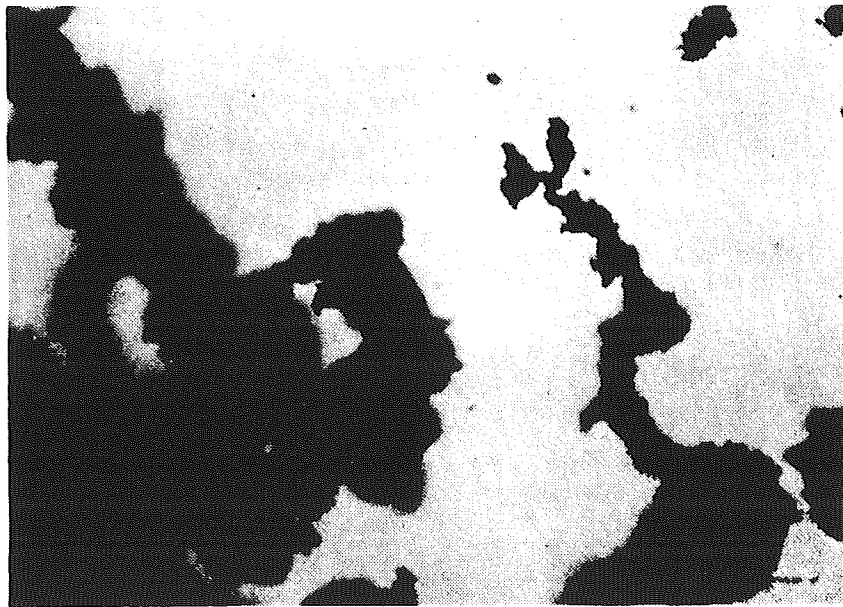


FIGURE 8. Microphotograph of Stycast 1090 ribbons.

Ribbon was produced under 500 grams/inch pressure on the blade. Stycast fragments into smaller particles when sawed at 100 grams pressure, producing a mixture of ribbon fragments and free particles.

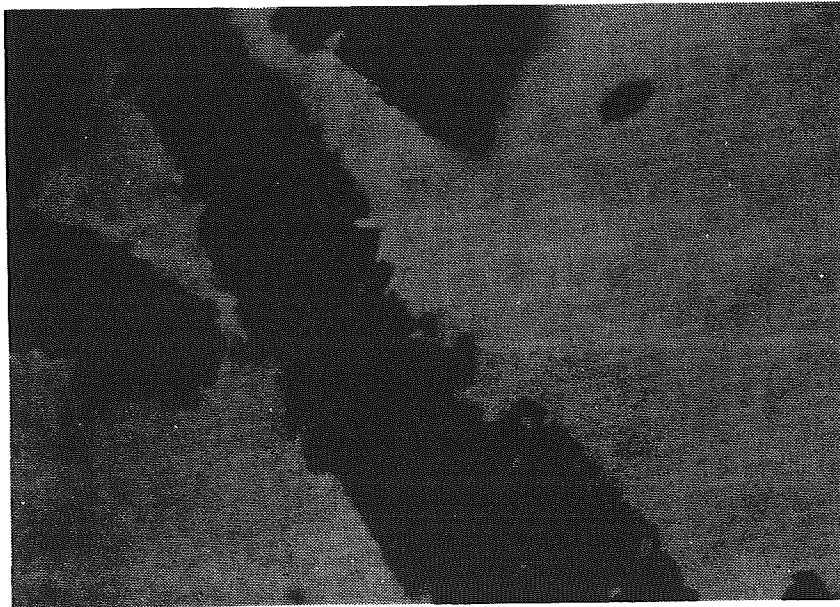


FIGURE 9. Microphotograph of Epocast 212/951.
Note fragments lying on edge.

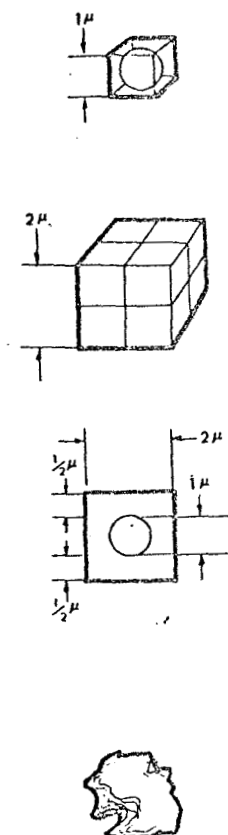
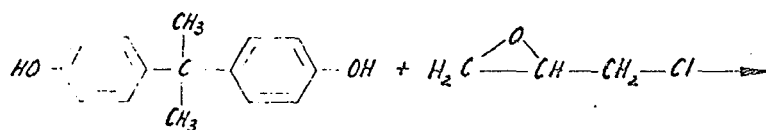


FIGURE 10. Theoretical model of entrapped spore.



BISPHENOL A

EPICHLORHYDRIN

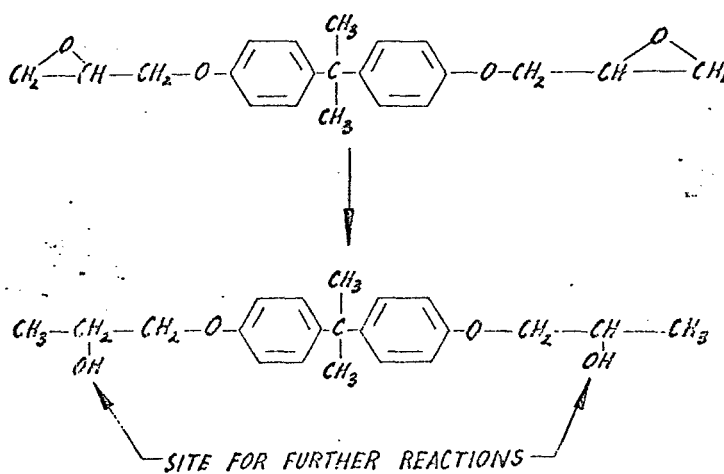


FIGURE 11. Synthesis of epoxy plastics.

THE EFFECT OF EPICHLORHYDRIN AND
EPOXY TYPE COMPOUNDS ON NUCLEIC ACIDS

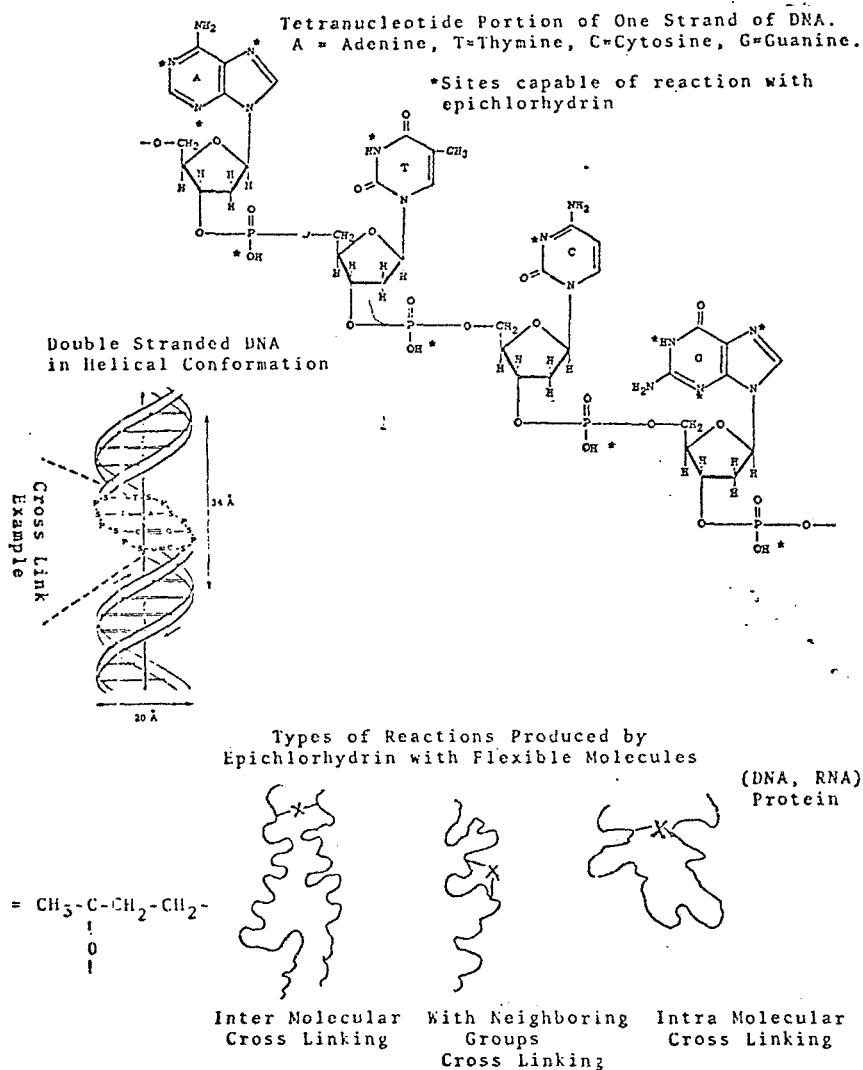


FIGURE 12. Interaction of epichlorohydrin with nucleic acids and proteins.

MICROORGANISMS IN SOLID MATERIALS

PHASES I, II, III, AND IV

(Including Mod. 2)

VOLUME II

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INTRODUCTION AND SUMMARY

In order to provide a high degree of reliability of microbial decontamination, it is essential that methods for the detection of viable microorganisms be developed and that these be as sensitive and reliable as possible. The detection of microorganisms within the interstices of solid materials represents a complex problem which may be approached conceptually from a number of directions. The detection methods which were studied and are reported in this document include: culturing, electron spin resonance, nonfluorescent staining, fluorescent staining, electrophoreses, and autoradiography.

A. Culturing

Culturing represents the most reliable method thus far devised for the detection of viable microorganisms. This method depends on the ability of the microorganism to undergo cellular division. There are, of course, deficiencies in the use of this method since physical and/or chemical trauma associated with the exposure of the microorganism from its location within the solid may result in a lack of ability to demonstrate the organisms' viability by culture methods. Thus, culture methods have long been established as useful methods for the detection of viable microorganisms and therefore will be used in Phases II, III, and IV.

B. Electron Spin Resonance

Electron spin resonance spectrometry involves the absorption of incident energy (usually in the microwave region) by unpaired electrons (free radicals) under the influence of a magnetic field. Electron spin resonance measures the magnetic moments of unpaired electrons and are somewhat analogous to the vibrational-rotational transitions in other forms of spectroscopy. Because free radicals occur in the process of metabolic

activity within cells, this technique was studied to determine its potential successfulness as an independent means of assessing the presence of microbial contamination in solid materials. Indeed it was found that differences existed between viable and nonviable microbial cells. Unfortunately, the order of magnitude of such differences is not sufficient to differentiate between other free radicals which may occur in a variety of nonliving solid materials (both organic and inorganic) of a wide variety of types. Since free radicals cannot be distinguished from those generated by small numbers of living microorganisms and inanimate solid particulates, this method was not considered to be of sufficient usefulness to warrant further investigation.

C. Nonfluorescent Staining

The cell walls of microorganisms contain chemical functional groups which vary in the nature and distribution of electrical charges. A wide variety of microbial stains adhere strongly to the cell walls and in some cases to the cytoplasmic constituents of microbial cells. The specificity and the degree of such staining is dependent upon the specific nature and distribution of the charge groups within the cell wall and the cytoplasm. In the application of this tool for the detection of microorganisms in solids, it is essential that the organism exposed from the solid be easily differentiated by microscopy from the debris. Not only is it of importance to determine the presence of microorganisms, but it is of greater usefulness to determine whether these microorganisms are viable or nonviable. Spores of B. subtilis var. niger, Clostridium sporogenes, and Ulocladium were obtained and their viability assessed by cultural methods. An aliquot of each of these microorganisms was subjected to the killing effects of dry heat, autoclaving, exposure to ethylene oxide, formaldehyde and chlorine gas. Subsequently, both the viable and nonviable organisms were stained and examined microscopically. No differences were noted of sufficient magnitude to distinguish between viable and nonviable microorganisms.

D. Fluorescent Staining

A number of fluorescent dyes bind strongly to microorganisms. When these microorganisms are examined under a microscope equipped with suitable optics, the background may be adjusted such that it appears uniformly black while the outlines of the microorganisms present a bright fluorescence. Thus, it would appear that this technique would be more useful than the nonfluorescent staining techniques since there are fewer interfering and confusing structures in the background. This method is only useful when the solid material and other debris from the pulverized solid do not possess chemically charged groups which bind the dye. In the latter case the solid as well as the microorganism would show fluorescence and the method would be useless.

E. Autoradiography

During the process of pulverizing solid materials, structural damage to the cell wall and the cytoplasm may take place. The effects of impact, drilling, crushing, and cutting may structurally alter the cell in such a manner that cell division may not be possible. Indeed in some procedures where pulverization is allowed to proceed to extreme limits the microorganisms may be fragmented. In order to demonstrate the possible viability of such microorganisms it is necessary to utilize methods which are capable of demonstrating viability which do not depend on cell division. The incorporation for radioactivity tagged weak beta emitting isotopes provide a means for determining potential cellular viability. The presence of the tagged metabolite may then be determined by the use of radioautography. The latter technique utilizes a sensitive fine grain photographic emulsion which is sensitive to weak beta emitting radiation. Weak beta rays interact with the photographic emulsion to produce opaque silver grains in a manner somewhat analogous to the effects of light on photographic emulsion.

The presence of grain patterns which are confined within the outlines of the microbial cell or fragments thereof would provide evidence of microbial contamination if adequate controls are included. When the opaque silver grain pattern develops in regions outside the cell wall area, it is possibly

due to absorption of the radioactive metabolite by the solid or inadequate washing. The resolution of this method is highest with the weakest forms of radiation capable of activating the silver particles in the emulsion. Also resolution is dependent upon the distance between the microorganism containing the radioactive metabolite and the photographic emulsion.

Spores of B. subtilis var. niger were incubated with solutions containing tritium labeled thymidine. In this experiment it was found that as many as three weeks of exposure of the microorganism to the tritium labeled thymidine were required to provide weakly positive results. The latter experiment was conducted on glass slides so that emulsion-organism distance was optimal. It is apparent that if this experiment had been conducted on a pulverized solid previously inoculated with microorganisms, the loss of resolution would likely have been sufficient to provide no reliable evidence of microbial labeling. Therefore, this method is not suited for the assay of viable or potentially viable microorganisms in solid materials.

Thus, it appears that of the methods discussed, culturing represents the most useful and accurate method for the assessment of viability. Under certain conditions fluorescent staining may provide evidence of the presence of both living and dead microorganisms and may have limited application for determining microbial contamination. None of the other methods show promise for potential usefulness.

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PHASE I. Studies on Various Methods for the Detection of Viable
Microorganisms Inoculated into Solid Materials

The objective of Phase I is to determine the effectiveness of a variety of methods for detecting viable cells. The following methods were explored in order to determine their possible usefulness as selective methods for detection of living microorganisms: culturing, electrophoresis, staining, autoradiography, and electron spin resonance. Since in the detection of viable organisms each method which could be applied might have specific advantages and disadvantages, it would be useful to have several independent methods for detecting viability. The detection of organisms by culturing is the most frequently used method of those listed. However, a devitalized cell may not grow under a specific set of cultural conditions and yet under other more ideal conditions may be capable of such growth. Thus, detection of viability by some other methods would be extremely useful. The potentially useful methods other than culture that were investigated in this phase include electrophoresis, autoradiography, and electron spin resonance.

I. PHASE I. STUDIES ON VARIOUS METHODS FOR THE DETECTION OF VIABLE MICROORGANISMS INOCULATED INTO SOLID MATERIALS

A. CULTURING

1. Introduction

a. Recovery from Solid Materials

The nature of solid substances imposes certain restrictions on the detection of microorganisms present within them. Microorganisms distributed on the outer surfaces are not as difficult to detect because of the availability of nutrients and lack of physical restrictions for cellular division to take place. Organisms located in the internal regions of solids must be freed to the extent that they are available for microscopic or cultural detection. The methods employed to achieve this may involve the use of one or more of the following principles: (1) dissolving in suitable solvent, and (2) pulverization.

In the case where the solid is dissolved, the theoretical likelihood of detecting contamination by microorganisms may be rather high. A major deficiency of this method is the toxicity of most plastic solvents to the microorganisms. Although it may be possible to detect the presence of such organisms, the likelihood of their demonstration in culture may be extremely low. Organic solvents are commonly used to disrupt permeability of microorganisms, and it would be anticipated that this generally takes place in the process of dissolving solids. The most useful application of dissolving techniques would seem to reside in microscopic scanning of stained residue obtained from the solubilized solid. Unfortunately, however, most solids do not redissolve following polymerization.

b. Nitrogen and Carbon Compound Requirements

Detection of viable microorganisms by virtue of their capacity to grow is one of the most useful methods for assessing sterility. The ability of the microorganism to assimilate nutrients from the culture medium, to convert

these nutrients to energy and structural materials for the cell, to maintain the cell's internal environment, and finally the ability of the cell to divide and provide viable daughter cells represent the requirements for cultural methods. Some bacteria require only the presence of certain trace minerals, carbon dioxide, and an inorganic source of nitrogen. Others require only a single simple form of carbon compound and utilize nitrogen from the atmosphere in place of more complex nitrogen compounds. On the opposite end of this spectrum are microorganisms which require vitamins, all seventeen natural amino acids and even traces of as yet undefined growth promotion substances. Most microorganisms lie somewhere between the two extremes.

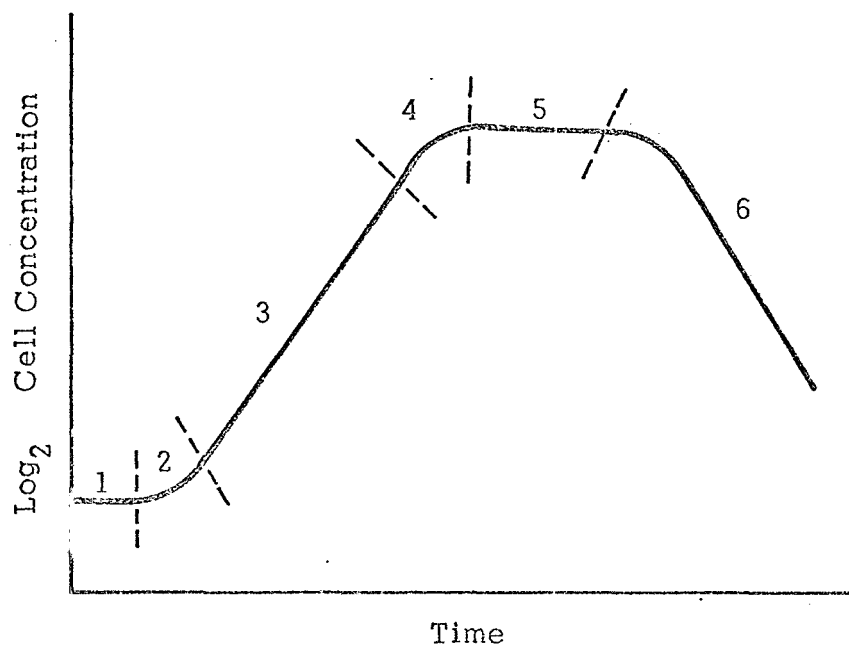
c. Growth Phases

Microorganisms pass through six distinct phases in their cultural life cycle. In the first phase cell division does not occur; however, the organisms may increase in size and accumulate metabolites. This condition exists in the lag phase (Figure I-1). A period of increasing growth rate takes place in the second or "acceleration" phase. The organisms during this phase of growth are dividing at a more rapid rate than during any of the other phases. The logarithmic phase is characterized by a constant or exponential growth rate which is followed by the retardation phase in which the growth rate is decreasing. The stationary phase is characterized by a steady state situation in which the rates of cell division and cell death are approximately equivalent. Finally, there is a phase of decline in which the number of cells dying exceed those dividing.

d. Concentration of Nutrients, Ionic Strength and Salt Concentration

The effects of concentration of nutrients on the rate and extent of growth are depicted in Figure I-2. Ideally in the absence of toxicity growth media should contain such large excesses of growth factors and metabolites that the extent of growth is not limited by their concentration.

Microorganisms are also capable of surviving under extremes of physical and chemical environments. Halophilic organisms, for example, are



Section of Curve	Phase	Growth Rate
1	Lag	Zero
2	Acceleration	Increasing
3	Logarithmic	Constant
4	Retardation	Decreasing
5	Stationary	Zero
6	Decline	Negative (more cells dying than reproducing)

Figure I-1. Growth Phases and Cell Concentration

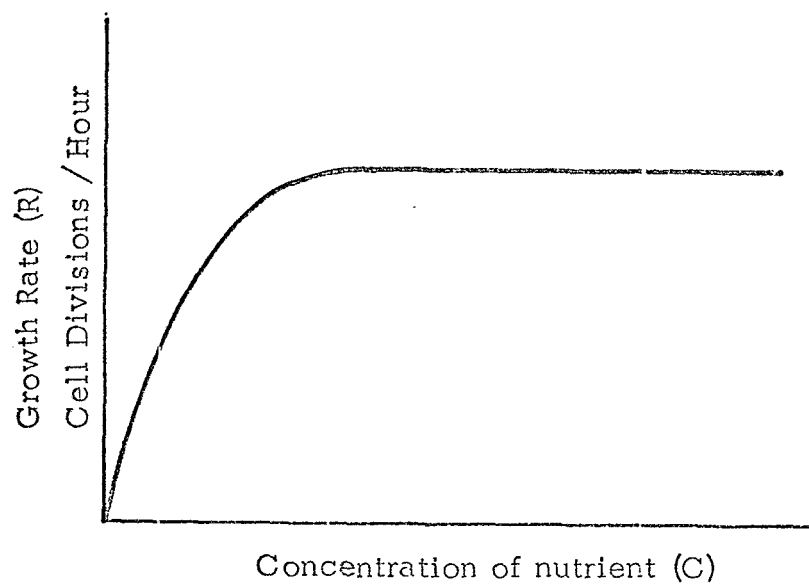


Figure I-2. Concentration of Nutrients

capable of growing in concentrated salt solutions. Some of these organisms have an absolute requirement for high salt concentration for growth. This environment generally decreases or inhibits the rate of growth of most other microorganisms. A wide variety of bacteria, on the other hand, do not grow well in the presence of even moderately low concentrations of salt. These organisms by virtue of their decreased tolerance to ionic strength may require extremely dilute growth media. The vast majority of microorganisms are able to grow at ionic strengths somewhere intermediate to these extremes.

e. pH and Physiological State

An equally important consideration is the pH of the growth media. Certain specialized bacteria are capable of withstanding the effects of and grow in the presence of strongly acid solutions. Organisms which oxidize sulfur to sulfuric acid are capable of growing in an environment which is strongly inhibitory to nearly all other microorganisms. At the opposite end of this spectrum are bacteria and fungi which accumulate basic substances in the course of growth or metabolism. Intermediate between these extremes are organisms which grow optimally at pH's in the vicinity of neutrality. Many of these organisms when growing anaerobically accumulate lactic acid as a terminal metabolic product. This accumulation causes a rapid progressive decrease in the growth rate. Likewise, organisms undergoing alkaline fermentation may eventually attain such a high pH that it interferes with growth. In these instances the presence of suitable buffers would resist such pH changes.

The pH of culture media is important as certain organisms grow only within certain limits of acidity and alkalinity. Most organisms seem to grow best at or around the neutral point, pH 7.0, but there are those which require a very acid pH. For example, certain fungi prefer pH 4.0-5.0, and Thiobacillus thiooxidans is active at pH 1.0. Brevibacterium ammoniae, responsible for napkinrash in babies, grows best at pH 9.0.

Sometimes advantage is taken of the ability of an organism to grow at an unusually high or low pH in order to isolate it. The media used in

recovering the Cholera vibrio from stools are made at pH 9.0, which permits the growth of the vibrio but inhibits that of most of the other organisms in feces.

The accumulation of toxic products results in a physiological condition in which the organism remains viable, but reproduces only after a prolonged lag period. After such organisms have been allowed to remain in a resting state with very small quantities of nutrients for prolonged periods of time a similar state of disability exists. This condition in either case is called the "staleing phenomenon." Such organisms are often more resistant to the effects of killing agents than organisms from the logarithmic phase of growth (Figure I-3).

f. Oxygen Tension

Some microorganisms are poisoned by the presence of oxygen in their environment. These organisms are called obligate anaerobes and, for the most part, are spore formers. In the spore stage the organism is highly resistant to the effects of dessication, oxygen tension, temperature, pH extremes, physical and chemical trauma. Conditions favorable for the proliferation of the vegetative form of these organisms, require that the oxygen be extremely low. A wide range of organisms are capable of growing under strictly anaerobic conditions even though these conditions may not be optimal. Such organisms which can grow in the presence of oxygen as well are called facultative anaerobes. At the far end of the spectrum are obligate aerobes which grow well only under high oxygen tension. These organisms are found in abundance in nearly all aerosol and soil environments.

g. Temperatures

Microorganisms grow optimally in temperature ranges between 20 and 40 degrees centigrade. Those organisms which proliferate in soil often show a growth optimum in the vicinity of 20 to 25 degrees centigrade. whereas those found in the environment of mammals show optima between 35 and 40 degrees centigrade. Certain organisms, however, grow optimally at temperatures higher than 40 degrees centigrade. These are thermophiles.

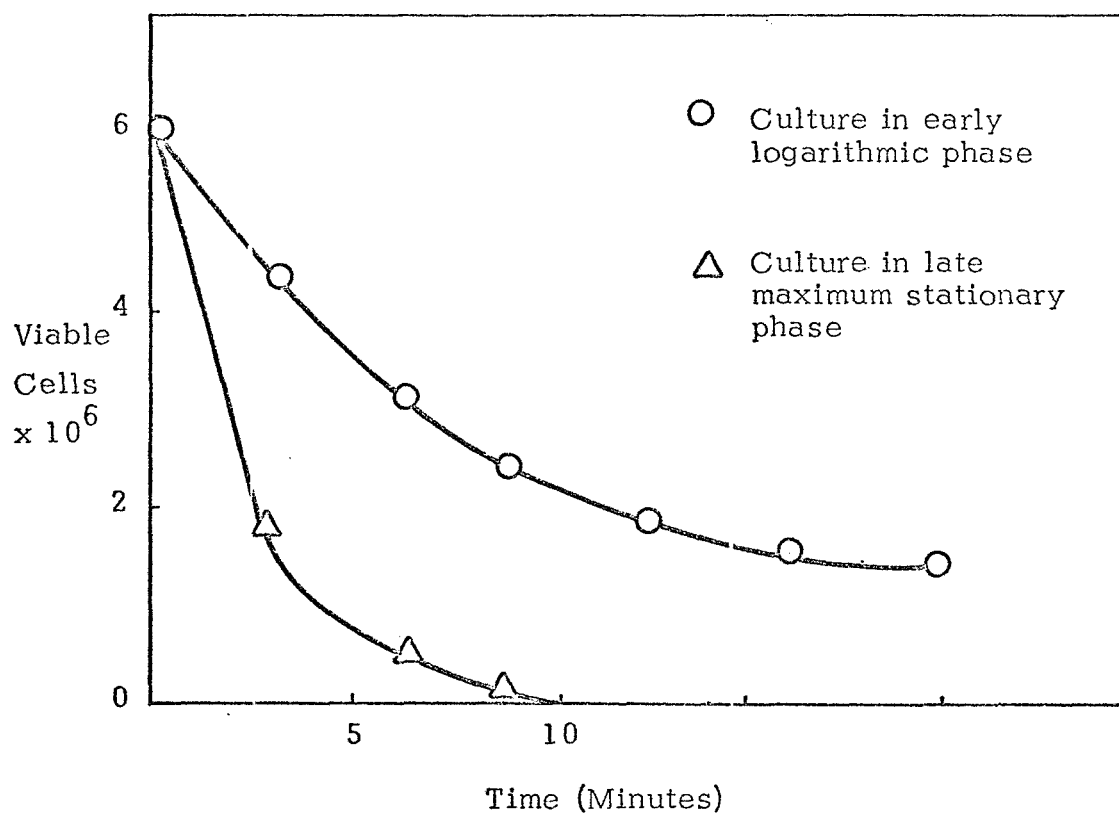


Figure I-3. Comparison between cells of young and old populations showing susceptibility to a lethal agent. Data graphed from raw data of Forbisher, 1962.

Not only can they grow at higher temperatures in the vicinity of hot springs and geysers, but they also show resistance to the effects of much higher temperature ranges.

Virtually all microorganisms can withstand low temperatures and even freezing for varying periods of time. Even though some organisms may be destroyed by the formation of ice crystals within their cell walls or by shearing effects of ice crystals as they are forming in the media, a significant percentage of all strains of microorganisms can be recovered following exposure to such temperatures.

Nearly all microorganisms can proliferate in liquid medium, although some grow well in the presence of very small quantities of moisture. The presence of moisture is particularly important when considering temperature effects on microorganisms. Resistance of spores to heat in high moisture environment is not nearly as great as that observed in the dried state. A well known protein, ova albumin, may be heated to 170 degrees centigrade in the dried state without becoming denatured. However, in the presence of water this protein is rapidly coagulated at 75 degrees centigrade. A second factor in heat resistance is manifested by the presence of colloidal substances. It is well known, for example, that the temperature required to kill lactic acid bacteria is markedly increased in cream or milk, as contrasted to water. It may be noted that the volume of most bacterial spores is roughly equivalent to one-tenth that of the vegetative form. This suggests that one reason for heat resistance exerted by the spore may be due to the concentration of the vegetative cytoplasm into a volume one-tenth that of its original volume, and hence a bio-colloid heat stability may be part of the explanation. Increased concentrations of calcium ion in the medium likewise leads to added heat stability. It has also been shown that fatty acids added to the growth medium help to impart increased resistance to heat.

2. Discussion

a. Limitations

The major limitation of culturing methods for determining sterility of

solids is that it measures only the ability of the organism to reproduce and does not measure its ability to metabolize independently of reproduction. It is conceivable that microorganisms can persist in solid materials for prolonged periods of time and yet fail to be detected by cultural methods because of deficiencies in the composition of the growth media, the procedures used to detect growth, the improper temperature or pH range, the presence of growth inhibitors which may be associated with the solid, or nonoptimal pulverization.

b. Requirements of Growth Media

Most commercially available culture media contain partially hydrolyzed proteins, peptones, and/or purified amino acids. Likewise, most of these media contain adequate quantities of vitamins. The growth media for any particular organism, however, may not be optimal in its concentration for any single constituent. It is possible for the media to be adequate in all constituents for most microorganisms and yet be marginal or deficient for a particular organism. Similarly, the growth of some organisms may be strongly inhibited by the presence of large quantities of certain media constituents.

Organisms which have been subjected to physical or chemical injury may lack the capacity for growing in an adequate medium due to cellular damage. This damage may be reversible in the case of exposure to a variety of chemical disinfectants (hexachlorophene and quaternary ammonia compounds). The latter two agents can induce sufficient damage to prevent the growth of a wide variety of microorganisms. The effects of this damage may be reversed by adding polyoxyethylene sorbitan mono-oleate and purified soybean lecithin. It is believed that these substances are assimilated by the injured organisms and that they reconstitute the damaged hydrophilic-hydrophobic cell membrane interface. Likewise, it is known that certain substances may be added to the growth media of microorganisms inhibited by ionizing radiation and that subsequent growth takes place. Little work has been done on attempts to reverse the effects of heat on microorganisms.

Although there are good theoretical grounds to support the view, at least some heat damage may be reversible.

c. Procedures Used to Detect Growth

The most common method of observing the growth of microorganisms is dependent upon visual perception of turbidity, pellicles, colonies, or floating particles. Usually such materials are stained and examined microscopically. This means of detecting growth is inadequate when such growth is slow or abortive. Detection precision can be increased by centrifuging fluid cultures which otherwise do not show growth, and examining microscopically the stained sediment.

B. ELECTRON SPIN RESONANCE

1. Introduction

Electron spin resonance spectrometry represents an application of a physical method for the study of biochemical phenomenon. Electron spin resonance involves the absorption of incident energy (usually in the microwave region) by unpaired electrons under the influence of a magnetic field.

a. Theory of Method

Magnetic resonance is the phenomenon of inducing transitions between unpaired electrons of differing energy levels. These energy transitions are analogous to vibrational-rotational transitions in other forms of spectroscopy. Electron spin resonance is concerned with the magnetic moments of unpaired electrons. Electrons spin like tops and being electrically charged create a magnetic field. The energy generated by this magnetism can be detected and measured in a magnetic field. If this spinning activated electron is also subjected to another magnetic field which is at right angles to the main magnetic field that is created, but which is pulsating, then the frequency of the pulse becomes the same as that of the frequency of precession. Then interaction will occur which will change the electron's orientation. When this change occurs, energy is absorbed. This absorbed

energy is detected in an electron spin resonance spectrometer.

b. Existence of Free Radicals in Living Systems

The first evidence that unpaired electrons associated with free radicals of metabolic origin were reported in freeze-dried samples of microorganisms, plant, and animal tissues. Because the free radical concentration of most microbial systems is very low (on the order of 10^{-7} to 10^{-5} M) extremely sensitive instrumentation is required. The volume of most electron spin resonance detection containers is on the order of 0.1 to 0.3 cm³. Electron spin resonance studies of oxidation reduction enzyme systems provide a possible means of detecting enzymes. Michaelis in 1940 suggested that although many enzymatic oxidation reduction processes appear to involve two electron transfers, electrons are actually transformed one at a time after the first step.

Carefully detailed electron spin resonance investigations of succinic acid dehydrogenase have been particularly useful in providing information regarding the role of free radicals in enzyme catalyzed processes (Figure I-4).

Major sources of unpaired electrons in living systems include:

- 1) Respiratory enzymes
 - a) flavins (flavin mono nucleotide, flavin adenine di nucleotides)
 - b) cytochromes b, b₁, C, C₁, a, a³
 - c) hemoglobin, myoglobin
- 2) Metalloflavo proteins (succinic acid dehydrogenase, aniline oxidase, aldehyde oxidase, nitrate reductase, sulfate reductase, aldehyde reductase)
- 3) Enzymes concerned with photosynthesis

In addition, an excited electron may travel through a continuum of fibrous proteins. It has been suggested that such proteins may link the insoluble outer fibrous proteins with the insoluble oxidation enzymes of the cell. Desoxyribonucleic acids (DNA) also may contain excited electrons

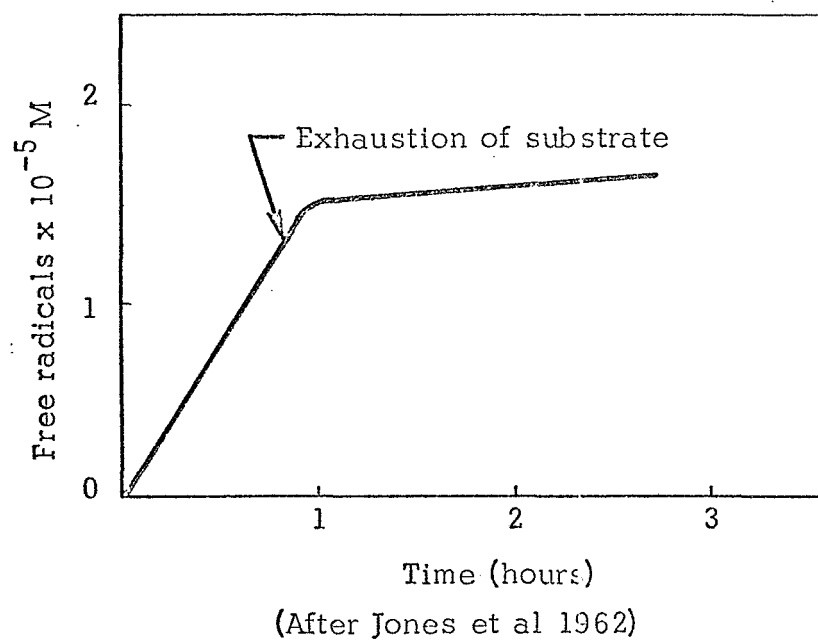


Figure I-4. Free Radicals Formed by
the Acid by Succinic
Dehydrogenase

following excitation. Biological systems carrying out either oxidative or reductive pathways would thus create a steady state stream of unpaired electrons. As would be expected when the living organism is no longer in the living state where unpaired electrons would be found. Unpaired electrons appear to be essential components of living organisms by virtue of their necessary role in biological oxidation and reduction mechanisms.

Other types of systems exist in which paramagnetic electrons may be demonstrated. Wool represents a material produced by a living system but which is a nonliving tissue and following electrical or light excitation, contains unpaired electrons. Szent-Györgyi in 1946 described the presence of unpaired electrons in gelatin dyed with various ionic dyes. The paramagnetic spectrum of hemoglobin is shown in Figure I-5. The spectrum of the amino acid glutathione is shown in Figure I-6.

2. Methods

In an exploratory test to determine whether the effects noted by Heckly and Dimnick might occur in other types of cells, the following steps were taken. Spores of B. subtilis were placed into each of several quartz tubes. These tubes were 9 inches long and 2 mm in diameter in order to fit the sample holder in the Varian EPR spectrometer. The tubes were sealed on one end but open to the atmosphere on the other. Except for those in one tube, the spores had been dried from suspension in acetone and were in the form of dry, unground, lumpy powder. They numbered 2.5×10^{11} spores per gram of dry powder and each tube contained about 0.1 gram of them. From the original container, the spores were sifted into a sterile beaker using a sterile fine-mesh screen sieve. By means of a small glass funnel they were then transferred into the quartz tubes. Yeast, in the form of dry active cells (Fleishmanns baking yeast) (approx. 5×10^9 cells) were similarly dispensed into quartz tubes.

Using a disposable glass syringe, 0.1 ml of the liquid phase of each of the biocides; ethylene oxide, ethylene imine, acrolein, acetone, or peracetic acid (40%) was placed on top of the organisms in the tube.

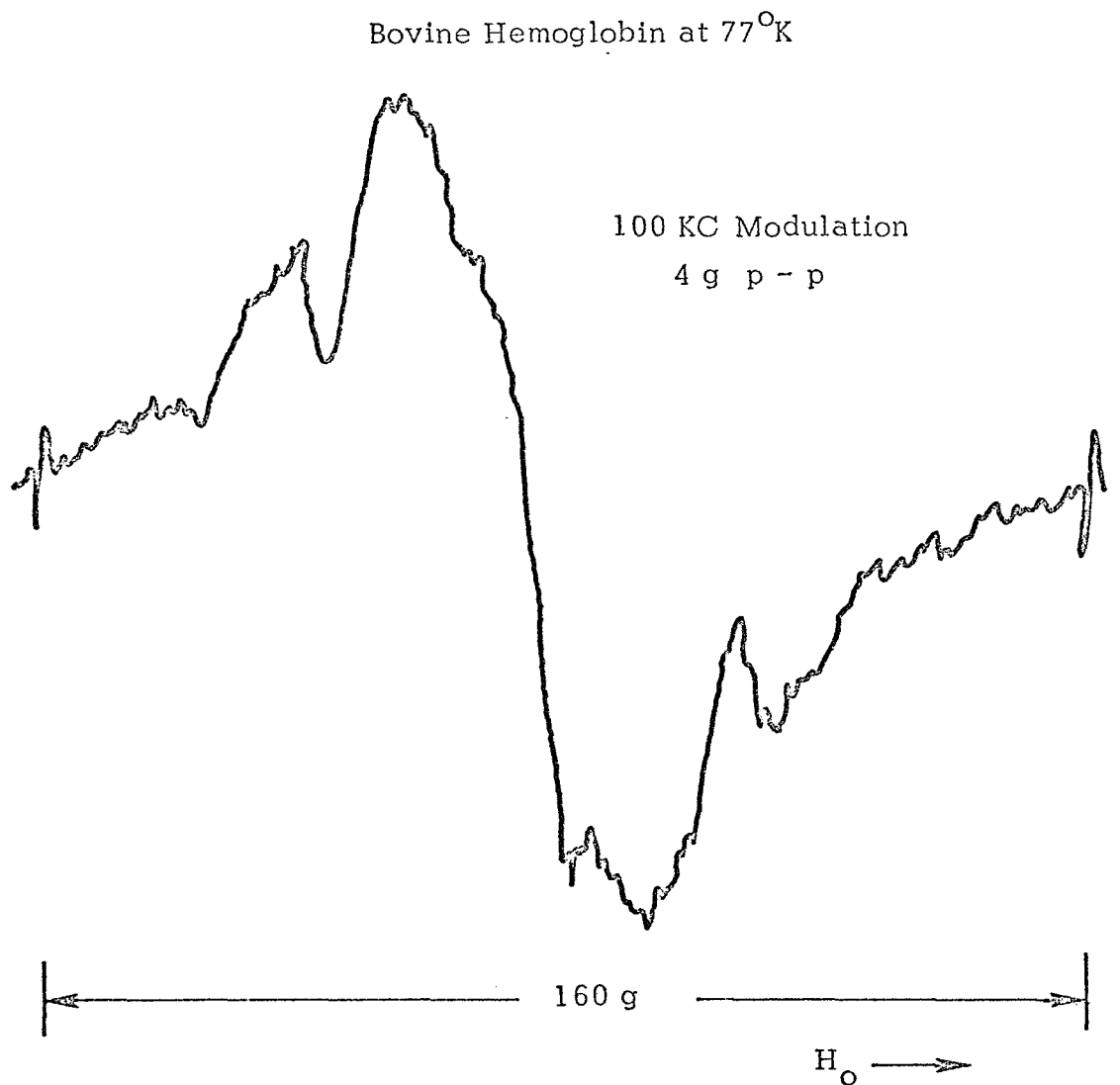


Figure I-5. First derivative trace of paramagnetic resonance absorption developed in bovine hemoglobin after ultraviolet irradiation at 77°K. Only the weak pre-irradiation absorption could be detected in this sample when it was warmed to room temperature. H_1 field was approximately 0.07 gauss.

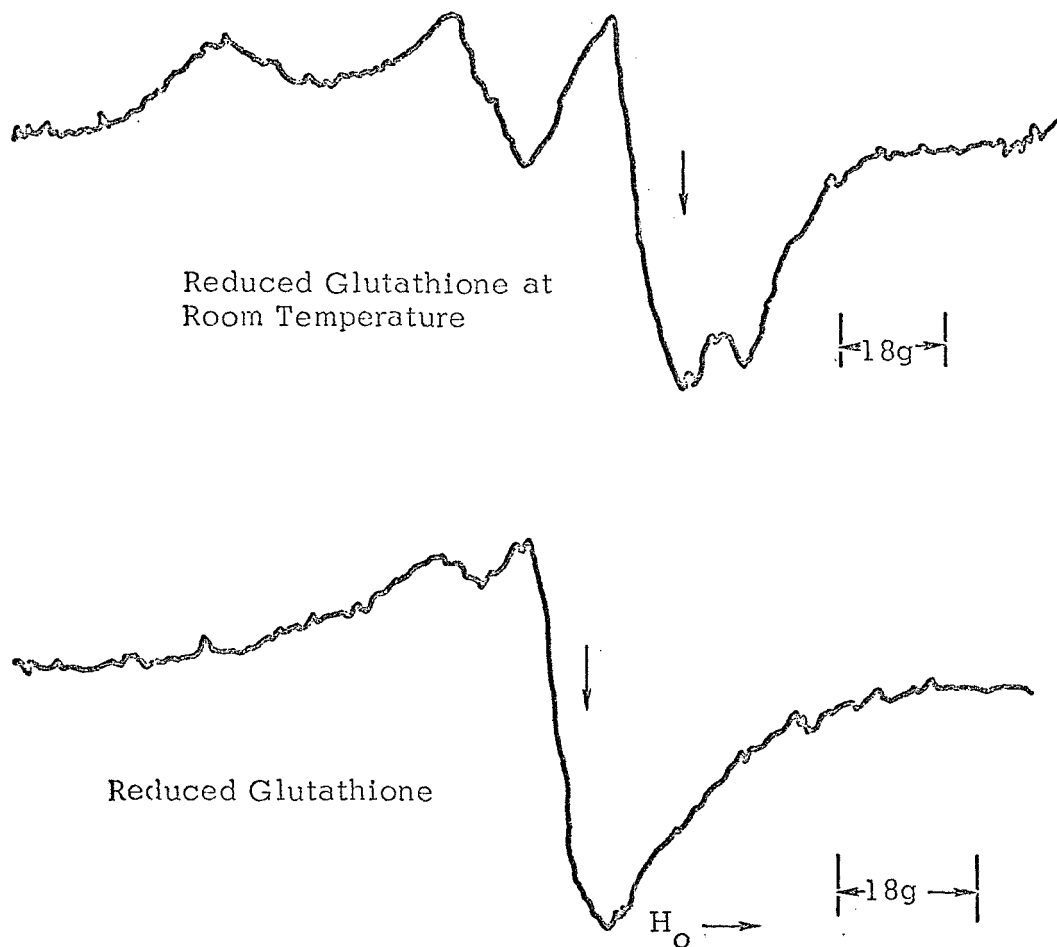


Figure I-6. First derivative traces of paramagnetic resonance absorption in reduced glutathione. This compound is a tripeptide composed of glutamic acid, glycine and cysteine. This compound, in the form of a dry powder, was placed in a tube and the tube was evacuated in an attempt to remove oxygen. The tube was then sealed and the compound was cooled to 77°K and irradiated with ultraviolet light. During the absorption measurements at 77°K, the 100 KC modulation amplitude was 0.6 gauss peak-to-peak and H_1 approximately 0.15 gauss at room temperature, the 100 KC modulation was 2.0 gauss peak-to-peak, the H_1 field strength was unchanged.

3. Results

The bacterial spores with and without ethylene oxide, chlorine, or ethylene imine gave a very strong signal (in excess of 1 millivolt). The yeast gave a weak signal which was the same whether or not they had been treated with biocide. Heating the tubes gently produced a darkening of the contents and an increase in signal. This same effect of heating (charring) occurs with sugar, however.

4. Discussion

A vast variety of materials derived from nonliving sources are also known to have unpaired electrons. Riehl (1955, 1956) has considered the energies, ϵ_1 , to transfer an electron from a molecule in a crystal lattice to its nearest neighbor in the lattice, and ϵ_2 to transfer an electron from a molecule to a distant site within a lattice. These quantified according to Lyons (1957) are:

$$\Delta \epsilon_1 = I - A e^2/r - P_{\mu}$$

$$\Delta \epsilon_2 = I - A - 2P_i$$

Here I is the ionization potential, A the electron affinity of the molecule in vacuo, e/r is the Coulombic energy of the ion pair, P_{μ} the polarization of the crystal due to the ion pair, and $2P_i$ the polarization of the crystal due to the separated ions. Due to the vast range of possible compound crystals and types of interactions in electronic components and component materials, it would be expected that paramagnetic signals could occur in nearly any range and could obscure signals found from living cells (due largely to paramagnetic species of carbon oxygen, sulfur, nitrogen, copper, iron, molybdenum and zinc).

5. Conclusions

Clearly, ESR, if it were effective in detecting the presence of living organisms in solid materials, would offer the possible advantage of non-destructive testing. Unfortunately, however, it is not possible to reliably

distinguish between living spores and dead spores, living yeasts and dead yeasts, or even charred material from living material. The difference in EPR signal emplitude in rapidly metabolizing, slowly metabolizing, and dead cells is not sufficiently large to provide data which would be useful even as a "screening" method.

Many solids derived from purely nonliving matter provide strong EPR signals. Examples of these materials are:

diphenyl picrylhydrazine
phthalocyanine
anthracene
dimethyl aniline-bromanil complex

In addition to the compounds on this list are many others including plastics mixed with metals.

EPR spectroscopy would not be useful as a method for the detection of living (or even nonliving) microorganisms in solid materials.

C. STAINING

1. Introduction

a. Theory of Methods

A wide range of dyes are available whose staining characteristics on microorganisms have been investigated. The binding affinity of the stain for the microorganism may reflect discrete and sometimes relatively specific chemical binding sites on the cell wall or cytoplasm of the organism. For example, Alican Blue is a dye which binds to acidic substances and is useful for showing the presence of acid mucopolysaccharides. A wide range of other dyes characterized by relatively strong and nonspecific staining properties are well known. The latter category includes most bacteriological stains, examples of which are methylene blue, Azure II, Gentian violet, and most of the triphenyl methane dyes. During the course of staining by these dyes, microorganisms are usually rendered nonviable.

As a rule, those organisms which are not stained by a particular dye may have cell walls which are protected by fatty or waxy coats, or the electrical charges on the dye molecule may be similar to that of the cell wall of the unstained organism and electrostatic repulsion prevents the dye from binding to it.

b. Potential Use with Solid Materials

When bacteriological stains are interacting with microorganisms, the binding affinities of the dyes for the organisms become highly critical. If the affinity of the dye for the microorganism is higher than that of the solid fragments of the materials, contrast will be apparent. This contrast is highly important and should under idealized conditions result in an unstained background of solid fragments and intense, characteristic staining of the microorganism. Theoretically one of the methods which could be applied to provide such staining characteristics involve the incubation of the pulverized solid with very dilute stain solution. When the microorganism binds the stain and the background of solid material fragments do not stain, the presence of microorganisms may be established. A second type of approach may be used in which the pulverized solid is treated with a more concentrated stain with the object of obtaining maximal staining of microorganism even though the pulverized solid fragments may stain intensely. This over-stained material may then be destained by treatment with a solvent in which the dye is very soluble. In some cases destaining may be accomplished by treatment with ether, acetone, or other nonaqueous solvents. Another approach to destaining is to use acid or alkaline solutions which reverse the binding of the dye to the surface of the solid particles, thus facilitating its removal with the solution leaving the microorganisms selectively stained.

One of the major limitations of dye staining technique is that the microorganism must possess a characteristic structure in order to differentiate it from stainable debris. Artifacts would thus obscure organisms which are distorted or which do not have distinctive shapes. Fungal elements and yeasts are usually less difficult to distinguish because of their more easily

distinguished intracellular detail and the presence of a well developed cell wall.

2. Methods

A mixed inoculum of Serratia marcescens, Bacillus subtilis, Clostridium sporogenes and Ulocladium was inoculated into solid rocket propellant and a solid epoxy plastic material (Maraset*). The inocula in both cases consisted of 10^3 of each organism/cm³ of the propellant or plastic. The solids were pulverized by sawing with a hack saw type blade.**

Approximately 100 mg of each of the solids were stained using the following stains:

- 1) Gram stain
- 2) 2.0% methylene blue
- 3) Alicant blue
- 4) Carbol fuchsin
- 5) Malachite green

Destaining was attempted with the following solvents:

- 1) Water
- 2) 2% HCl in water
- 3) Acetone-alcohol
- 4) Chloroform

Each of these destaining solutions were visually controlled by microscopic observation.

3. Results

The backgrounds of the stained specimens were unsatisfactory in all cases. It was not possible in most cases to clearly differentiate the

*Marblette Corp. 37-31, 30th St., Long Island City, N. Y.

**Blade No. 1218-3, Clemson Bros., Inc.

spores from the background debris. The appearances of the stained organisms are shown in Tables I-1, -2, -3. The background consistently was nonuniform in appearance. The conidia (spores of fungi) could be seen and differentiated in all stained preparations; however, scanning was tedious and many stained fragments could not be unequivocally distinguished from debris.

The propellant was opaque and stained intensely. Destaining did not lead to improvement of microscopic detection of microorganisms. The staining methods as applied in this study were not useful in the detection of microorganisms in solid propellant.

4. Discussion

Staining methods are useful for detecting microorganisms under certain specified conditions. If the background of the microscopic field is sufficiently uniform they may be easily differentiated from the debris. In the process of pulverizing solid materials, fragments of various sizes and shapes and possessing varying optical properties generally obscure the outlines of microorganisms and increase incidence of artifactitious interpretation is possible.

No differences were observed between stained viable and nonviable spores of B. subtilis.

5. Conclusions

Staining of pulverized solids using a variety of bacteriological stains was relatively ineffective for demonstrating the presence of bacteriological spores. Less difficulty was encountered in some instances in the Maraset plastic of detecting fungal hyphal and conidial structures than with the bacterial spores. Detection of microorganisms by staining of propellant was more difficult than with stained Maraset plastic.

Staining with the stains used in this study did not result in detectable differences between viable and nonviable organisms.

TABLE I-1

VIALE AND NONVIALE

Staining Characteristics of Spores of B. subtilis, var. niger

Stain	Spore Treatment	Color of Spores	Color of Vegetative Cells	Dispersion
Gram stain	Viable Untreated	Pink outlined in purple	Cells at periphery are pink	Well dispersed
	Heated dry	Blue to dark blue	Dark red	Clumped
	Autoclaved	Intense blue	Pink to red	Well dispersed
	Ethylene oxide treated	Same as for viable spores	Same as for viable spores	Well dispersed
	Chlorine treated	Intense dark green	Dark red	Clumps

TABLE I-2

VIABLE AND NONVIABLE

Staining Characteristics of Spores of B. subtilis, var. niger

Stain	Spore Treatment	Color of Spores	Color of Vegetative Cells	Dispersion
Malachite green spore stain	Viabile Untreated	Emerald green	None	Spores central well dispersed
	Heated dry	Intense dark green, black center	None	Clumped spores
	Autoclaved	Intense green	None	Well dispersed. Similar to viable cells.
	Ethylene oxide treated	Emerald green	None	Well dispersed

TABLE I-3
VIABLE AND NONVIABLE
Staining Characteristics of Spores of B. subtilis, var. niger

Stain	Spore Treatment	Color of Spores	Color of Vegetative Cells	Dispersion
Carbol fuchsin spore stain	Viable Untreated	Red	Light pink	Well dispersed
	Heated dry	Intense dark red	None	Clumped
	Autoclaved	Intense dark red	Light pink	Well dispersed
	Ethylene oxide treated	Same as for viable spores	Same as for viable spores	Well dispersed
	Chlorine treated	Intense dark red	None	---

D. FLUORESCENT STAINING

1. Introduction

a. Theory of Method

Using light microscopy and nonfluorescent dyes the background of the microscopic field is bright and contains the detailed and confusing outlines of opaque or semi-opaque solid fragments which may or may not stain the same color as the organism.

Microorganisms may be detected within solids more readily by the use of fluorescent staining. Organisms stained with a fluorescent dye may be visualized microscopically using an ultraviolet light source. The number of fluorescent dyes which have proven satisfactory for the staining of microorganisms are much smaller than those nonfluorescent dyes used for routine bacteriological staining purposes. However, there are distinct advantages to the use of ultraviolet fluorescent microscopy. The fluorescent light source system may be adjusted to provide a microscopic background field which is essentially black. The particles of solids which do not take up the fluorescent stain therefore would not be visualized. Ideally, the background should be as nearly black and uniform as possible. The organism which has taken up the fluorescent dye would appear as a bright fluorescent structure against a contrasting dark or black field. This, of course, facilitates the task of detecting small numbers of organisms in a pulverized sample.

Ideally, in the evaluation of the contamination level of solid materials, staining procedures should be able to detect relatively small numbers of viable cells, preferably as few as one. Such requirements indicate that the ultraviolet fluorescent technique should be best suited to the job. For this reason, most of the literature survey and the subsequent experimental work emphasizes ultraviolet fluorescent techniques.

b. Differentiation Between Living and Dead Cells

Very few reports were found in the literature in which the use of stains or dyes to distinguish viable from nonviable microorganisms was

described. Only one was found that was directly related to viability of microorganisms (Meisel 1961). Meisel reported that the fluorescent dye, primuline, could be used to distinguish between living and dead microorganisms. The report of the experimental procedures was somewhat brief but it did state that dead cells fluoresced much more intensely than did live ones. This effect was demonstrated by using live organisms in the dye on a microscope slide and heating the slide while it was under observation. As the heat killed the cells they showed intense fluorescence. The effects were reported for vegetative cells.

Several investigators have reported that differentiation between living and dead cells, other than microorganisms, was possible with the fluorescent dye, acridine-orange. Yurtsev (1960) used the dye to determine pollen viability in certain cereals. Vinegar (1956) reported differential staining of living and dead ascites tumor cells with acridine-orange. This dye has been used to distinguish between cancerous and noncancerous cells and is the basis for rapid screening techniques on cervical and vaginal smears. Van Niekerk (1962) reported a procedure using acridine-orange to detect pathogenic vaginal flora in vaginal smears. Both bacteria and protozoa were stained by the dye. There are several descriptions (Riva, 1962, Anon., Stain Tech., 1962) of the use of acridine-orange for staining of tissue sections and for exfoliative cytology studies. While there were some minor modifications in all the reported procedures using acridine-orange, all used dye dilutions of from 1:5,000 to 1:10,000 in an acid (pH 3 to 5) buffer.

Oginsky and Umbreit (1955) point out that cells of certain microorganisms lose acid fastness (stain not removable by acid solution) when subjected to mechanical damage. The possibility is suggested that loss in acid fastness might also be a consequence of loss in viability. One of the staining procedures for bacterial spores is based on an acid fast technique using carbol fuchsin (not a fluorescent dye). Auramine-O is a fluorescent dye used to stain acid fast tubercle bacilli and might be useful in distinguishing between viable and nonviable spores. These spore-staining and acid-fast staining techniques are a portion of standard laboratory procedure (Conn 1957).

Another potentially useful fluorescent material is the antibiotic, tetracycline. While not considered to be a biological dye, this antibiotic is absorbed by many types of cells and does fluoresce in ultraviolet light. This material has been used to identify growing bone (Hattner 1962) debris in the region of cancerous growths (Milch 1961; Vassar 1962), certain parasites (Tobie 1960), and the mitochondria of cells (DuBuy 1961) by the fluorescent technique. One theory of the action of the tetracyclines is based upon the binding of calcium ion (Anon., Chem. Eng. News, 1962), and action upon enzymes which require calcium similar to that of some chelating agents. Riemann (1961) has shown that germination of spores can be greatly stimulated by the use of chelating agents in the presence of the proper calcium concentration. It is possible that in the presence of calcium, tetracycline could act as a spore germination stimulant with the result that viable spores would have a greater uptake of tetracycline than nonviable ones. If this were the case, a possible staining procedure for distinguishing viable from nonviable spores might result.

Other fluorescent biological dyes are available (Anon., Reichert, 1963) for staining microorganisms. The fluorescence and absorption spectra for many of these dyes are available (Porro 1963).

c. Fluorescent Antibody Technique

One of the fluorescence techniques which has been the subject of extensive investigation since its introduction in 1941, is that of the fluorescent antibody technique. In one form of this technique, an antibody is prepared, in some appropriate laboratory animal, against a specific microorganism. The antibody is labelled with a fluorescent molecule. When the labelled antibody comes in contact with the antigen (in this case, some portion of the specific microorganism) the antigen-antibody reaction site is marked with the fluorescent label. This technique, as well as several modifications, has been used successfully in the rapid and quite specific identification of microorganisms. It is sufficiently sensitive that a single microorganism can be detected.

2. Methods

a. Fluorescent Dyes

The four fluorescent dyes selected for investigation were tetracycline (or one of its derivatives), primuline, acridine-orange, and auramine-O. No staining procedures using tetracyclines were found in the literature so that preliminary studies to develop an effective procedure were made. Some exploratory experimentation with primuline was also necessary. Procedures for acridine-orange and auramine-O were taken directly from the literature.

b. Staining Procedures Used

(1) Tetracycline. Two tetracycline compounds were selected for the initial screening, tetracycline itself and dimethylchlorotetracycline*. The latter compound gave the stronger fluorescence and was used in subsequent procedures. A 1/3 fractional factorial design was used with the following factors being investigated at three levels: pH of the buffer used to dissolve the dye, CaCl_2 concentration, exposure time of the microorganisms (B. subtilis spores) to the dye- CaCl_2 mixture, and the pH of the buffer used to mount the coverslips on the slides. On the basis of this experiment it was found that the intensity of the fluorescence of the spores was neither dependent on the pH of the buffer used to dissolve the dye nor on the CaCl_2 concentration (including none). There was a small effect of exposure time (one hour being best), and a strong effect of the pH of the mounting buffer. In the latter case, the more alkaline buffers were better, pH of 9.0 being the highest used.

A second experiment was performed in which the dye concentration and the type of buffer for coverslip mounting were the primary factors. Two buffer systems were used, one aqueous and one with glycerine. The results indicated that the concentration of the dye was not a critical factor. The glycerine buffer (pH of 9.0) was considerably better, however, than an aqueous buffer of the pH.

*These materials were donated by Lederle Laboratories as Achromycin and Declomycin, respectively.

On the basis of these two experiments, the following procedure was used:

1. Prepare an 0.5% solution of Declomycin in Sorensen's phosphate buffer, pH of 7.0.
2. To one ml of this dye solution add three drops of a B. subtilis spore suspension containing approximately 10^9 spores/ml.
3. Incubate for one hour at 37°C .
4. Centrifuge, remove, and discard the supernatant.
5. Resuspend the centrifuged spores in glycerine buffer, pH 9.0. This buffer is prepared by adding 9.8 parts of glycerine to 0.2 parts phosphate buffer, pH 9.0.
6. Place one or two drops of the suspension on a microscope slide, cover with a coverslip, and examine with ultraviolet light using oil immersion objective.

(2) Primuline. Meisel (1962) gave very little information on the preparation of the dye. Primuline is soluble in alkaline solutions and moderately insoluble in acid. Two pH's, 7.0 and 9.0, were selected for study and two primuline concentrations were used, 1:10,000 (one gram in 10,000 ml buffer) and 1:100,000. Because of the relatively small number of variables, no preliminary experiments were made. The following procedure was used:

1. Prepare four solutions of primuline of 1:10,000 and 1:100,000 dilution of phosphate buffer, pH of 7.0 and 9.0.
2. To one ml of each solution add 3 drops of the spore suspensions being studied (approximately 10^9 spores/ml).
3. Incubate 30 minutes at 37°C .
4. Centrifuge, remove, and discard supernatant.
5. Resuspend spores in phosphate buffer, pH of 4.4.

6. Place one or two drops of suspension on microscope slide, cover with coverslip, and examine.

(3) Acridine-Orange. The procedure of Van Niekerk (1962) was used and is as follows:

1. Prepare smears of spore suspensions on microscope slides and allow to air dry.
2. Fix smear in 95% ethyl alcohol and diethyl ether (1:1) for 10 minutes.
3. Pass slide quickly through rinses of 80, 70, and 50% ethyl alcohol.
4. Leave in distilled water for 2 minutes.
5. Put in citric acid-disodium phosphate buffer, pH of 3.8, for 3 minutes.
6. Stain in 0.01% acridine-orange in pH 3.8 buffer for 3 minutes.
7. Put in clean buffer, pH of 3.8 for 4 minutes.
8. Mount coverslip with same buffer.
9. Examine.

(4) Auramine-O. The procedure of Richardson and Miller (Conn 1957) was used and is as follows:

1. Prepare solution "A"

Auramine-O (94% dye content)	0.1 gm
Liquified phenol	3 ml
Distilled water	97 ml

2. Prepare solution "B" (must be freshly prepared)

Ethyl alcohol (70%)	100 ml
Conc HCl	0.5 ml
NaCl	0.5 gm

3. Prepare smears and air dry.
4. Stain in solution "A" for 2-3 minutes.
5. Wash in tap water.
6. Destain in solution "B" for 3-5 minutes.
7. Dry slide and examine with high dry objective.

(auramine-O does not fluoresce in solution).

c. Spore Suspensions

Viable spore suspensions were prepared by dry B. subtilis spores. Nonviable spore suspensions were prepared by exposing dry viable spores to ethylene oxide, autoclaving, dry heat, and chlorine gas. Viability and nonviability were verified by culturing on Trypticase soy agar plates. Final concentrations used for the staining procedures was approximately 10^9 spores/ml.

d. Microscopy

Ultraviolet fluorescent microscopy was performed using an American Optical binocular microscope equipped with a dark-field condenser. Objectives used were either a 43X high dry or a 97X oil immersion with an integral iris diaphragm. Oculars were 10X. The light source was a Leitz Model 250 using an Osram HBO 200 high pressure mercury vapor lamp. Filters available for the light source were a 4 mm red suppression filter, BG-38, a 2 mm heat absorbing filter, KG-1, a 2 mm ultraviolet fluorescence filter, UG-1, and a 3 mm blue fluorescence filter, BG-12. Available barrier filters were Wratten 2A, 2B, 2C, and 15G. For all of the studies the following combination was used: 2 mm KG-1, and 2 mm UG-1 lamp filters and a Wratten 156 barrier filter.

3. Results

a. Tetracycline

The first experiment compared the fluorescence of viable and

nonviable spores. Duplicate slides were used. Fluorescence intensity was judged semiquantitatively as + through ++++.

TABLE I-4

Tetracycline Fluorescence of Viable and Nonviable Spores of B. subtilis var. niger

Spore Suspension	Replicate	Fluorescence
Viable	1	++++
	2	++++
Autoclave killed*	1	
	2	
Dry heat killed**	1	++++
	2	++++
Chlorine killed**	1	+++
	2	+++
Ethylene oxide killed	1	++++
	2	++++

*spores failed to centrifuge

**spores markedly distorted

These results indicate no significant difference between the staining of viable and nonviable spores. In order to check the possibility that staining differences, if present, might be more apparent for short incubation times, a second experiment was performed in which incubation periods of 1, 5 and 15 minutes were used.

TABLE I-5
Effect of Incubation Time on Tetracycline Fluorescence
Staining of Spores of B. subtilis var. niger

Incubation Time	Spore Suspension	Fluorescence
1 minute	Viable	++
	Autoclaved	++
	Heated dry	+++
	Chlorine treated	++
	Ethylene oxide treated	++
5 minutes	Viable	++
	Autoclaved	++
	Heated dry	+++
	Chlorine treated	++
	Ethylene oxide treated	++
15 minutes	Viable	+++
	Autoclaved	+++
	Heated dry	++++
	Chlorine treated	+++
	Ethylene oxide treated	+++

Although there appears to be slightly greater fluorescence with the dry-heat killed spores than with the others, the difference is not sufficient enough to be the basis of a reliable differentiation technique.

b. Primuline

For the primuline experiment, spore suspensions were limited to viable and dry-heat killed. Two primuline concentrations and two buffer pH's were tested.

TABLE I-6

Effect of Primuline Concentration and pH on Fluorescence Staining Intensity of Viable and Nonviable Spores of B. subtilis var. niger

Primuline Concentration	Buffer pH	Spore Suspension	Fluorescence
1:10 ⁴	7.0	Viable	++
		Heated dry	+++
	9.0	Viable	++
		Heated dry	+++
1:10 ⁵	7.0	Viable	++
		Heated dry	+++
	9.0	Viable	++
		Heated dry	+++

Here again, there was a slightly greater fluorescence with the heat killed spores than with the viable ones. The differences, however, were not great enough by this procedure to permit a reliable differentiation between viable and nonviable spores.

c. Acridine-Orange Fluorescent Staining

The results are as follows:

TABLE I-7

Spore Suspension	Fluorescence
Viable	+
Autoclaved	+
Heated dry	++
Chlorine treated	+
Ethylene oxide treated	+

d. Auramine-O

The results are as follows:

TABLE I-8
Auramine-O Fluorescent Staining of Viable and
Nonviable Spores of B. subtilis var. niger

Spore Suspension	Fluorescence
Viable	+++
Autoclaved	+++
Heated dry	+++
Chlorine treated	+++
Ethylene oxide treated	+++

No differenced between viable and nonviable spores was found.

4. Conclusions

- a. No significant, practical differential staining reactions were found for any of the four staining procedures tested.
- b. There appears to be a slight increase in the fluorescent intensity of dry-heat killed spores compared to viable spores or to spores killed by other means.
- c. Fluorescent intensity for the above procedures was greatest with tetracycline, intermediate with primuline and auramine-O and least (weak) with acridine-orange. All procedures stained both spores and vegetative forms (some vegetative forms were present in the spores suspensions).
- d. Though no distinction between viable and nonviable contamination inside solids, through staining techniques, appears to be possible, detection of the contamination is quite possible and feasible for many solid materials. In combination with culturing, staining can increase the sensitivity of the detection technique

when the metabolizing cells act on ingested stain (Salberg 1960).

- e. In none of the fluorescence techniques nor the conventional staining techniques described in Table I-1 did the ethylene oxide killed spores appear different from the viable spores.

E. ELECTROPHORESIS

1. Introduction

a. Theory of Electrophoresis

Electrophoresis is a method of separating particles, microorganisms and macromolecules which depends upon their electrical charges and molecule shape and weight. When particles with an electric charge are placed in an electric field they will move toward the electrode possessing an opposite electrical charge, (Figure 1-7). The direction of migration is determined mainly by the charge in the particle. Particles with different charges will move with different velocities and thus can be separated from one another.

b. Potential Application for Separation of Living from Nonliving Cells

Life processes affect the electric charge-to-mass ratio of cells. The life processes going on in the living cell tend to maintain electric charges of characteristic types and magnitudes for particular cells. When populations containing both living and dead cells are suspended in a fluid in an electric field the two types may exhibit different mobilities. This technique if it were effective might be useful in determining death-rate mechanisms because in any particular sample it is almost impossible to observe all the cells, both living and dead. The ratio of cells exhibiting the two different electrophoretic mobilities would be measured.

The distance through which a particle moves under the influence of an electric current is proportional to the time and to the electrical potential gradient at the site where the particle is. The potential gradient in turn depends upon the current, the conductivity of the solution, and the cross-sectional

Functional groups which appear in
cell membranes and cell walls

Approximate pK value
(pH at which 50% of
functional group is ionized)

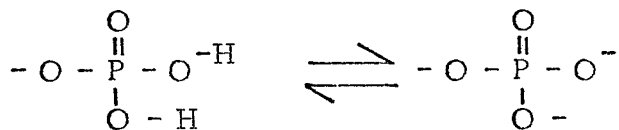
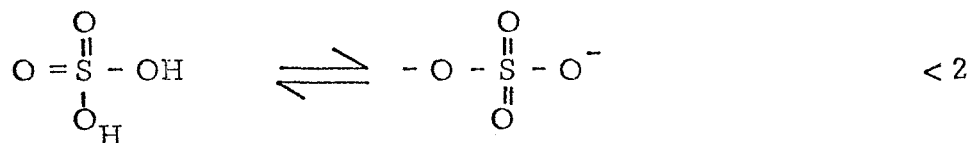
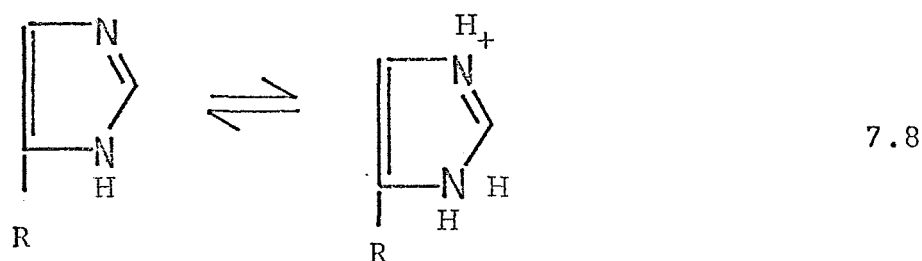
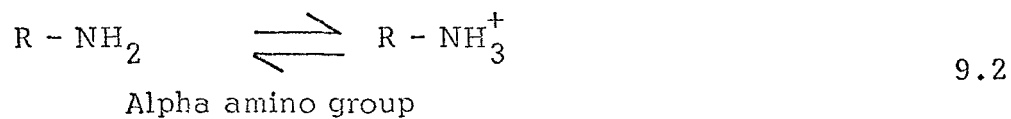
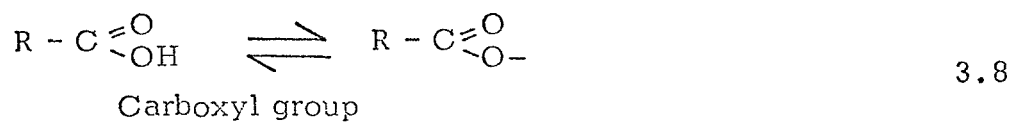


Figure I-7. Theory of Electrophoresis

area of the solution at this point. The proportionality constant is known as the electrophoretic mobility.

2. Methods

a. Description of Apparatus

A standard rectangular microelectrophoresis cell was utilized in the study in which the mobility of the cells was observed by a microscope with a total magnification from 200 to 1000X. The velocities were determined by focusing on a given particle and measuring the time required for the particle to traverse a given distance using a calibrated ocular micrometer. The microscope was focused at one of two planes in the microelectrophoresis cell. These two planes are referred to as the stationary planes. In an electrophoresis cell of this type the observed velocity of the particles will be composed of two components. One component is due to the true electrophoretic forces on the particle and the other is due to a motion of the fluid itself within the electrophoretic cell (the electro-osmotic streaming effect). The latter phenomenon is due to the effect of the electrical field on the suspending fluid. For a closed cell, as is used for electrophoresis studies, the velocity due to electro-osmotic streaming is a maximum on the centerline in one direction and is in the opposite direction along the walls.

At some plane between the cell center line and the wall the streaming velocity is zero. These are the stationary planes. The motion of particles in these planes is due solely to the electrophoretic mobility and these are the planes upon which the microscope must be focused. The isoelectric point is determined by finding the pH of the suspending fluid for which the particle velocity in the stationary plane is zero. This was done by measuring the velocity for a range of pH's on either side of the isoelectric point and plotting velocity against pH. The pH where the curve crosses the zero velocity line is the isoelectric point. (Figures I-8 and I-9)

b. Preparation of Organisms

Bacillus subtilis spores were killed by the following treatments:

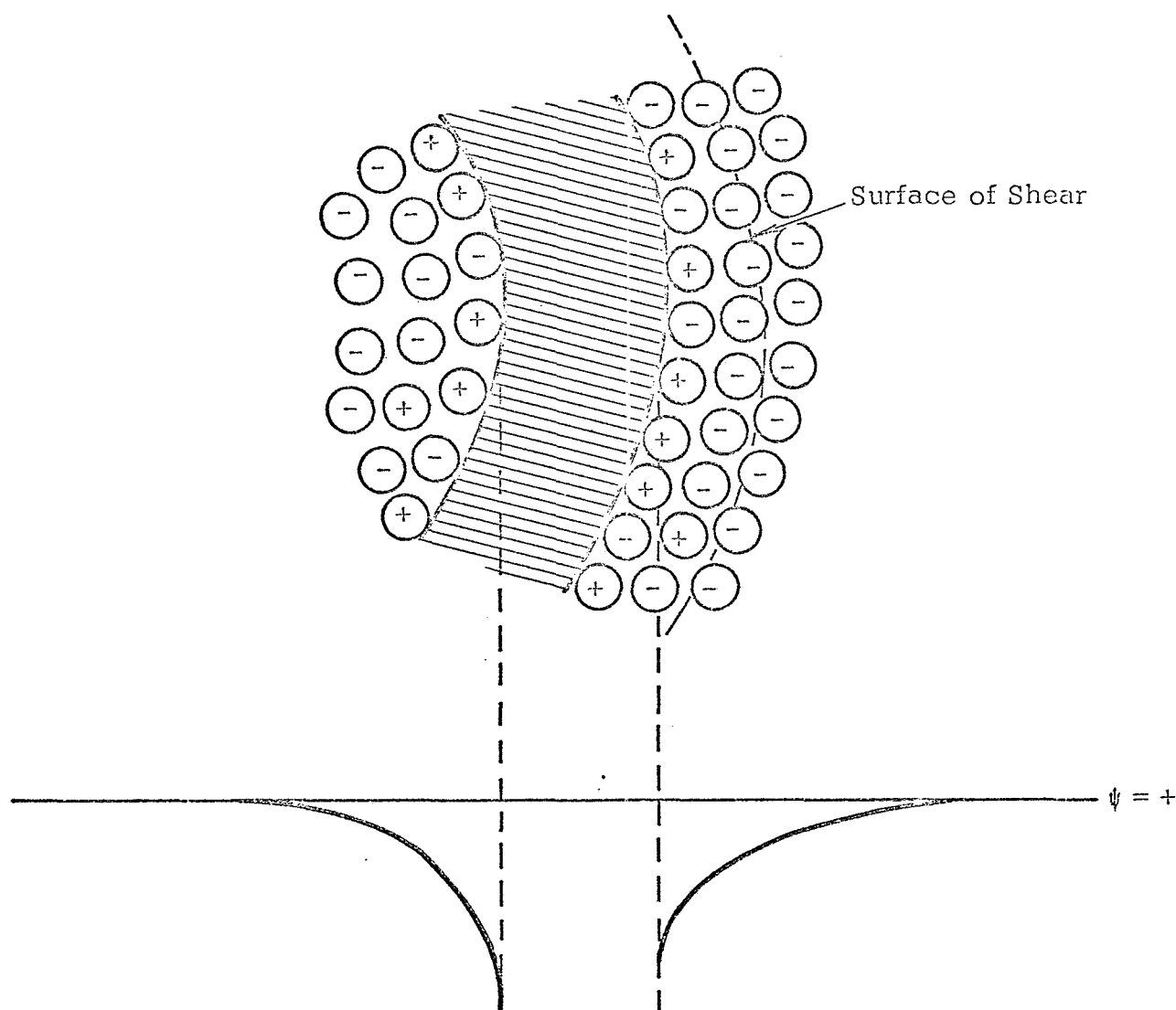


Figure I-8. Surface charge potentials are positive on both sides of the cell membrane. This occurs over a range of pH values in which amino groups are ionized and are more numerous than carboxyl or other acidic functions.

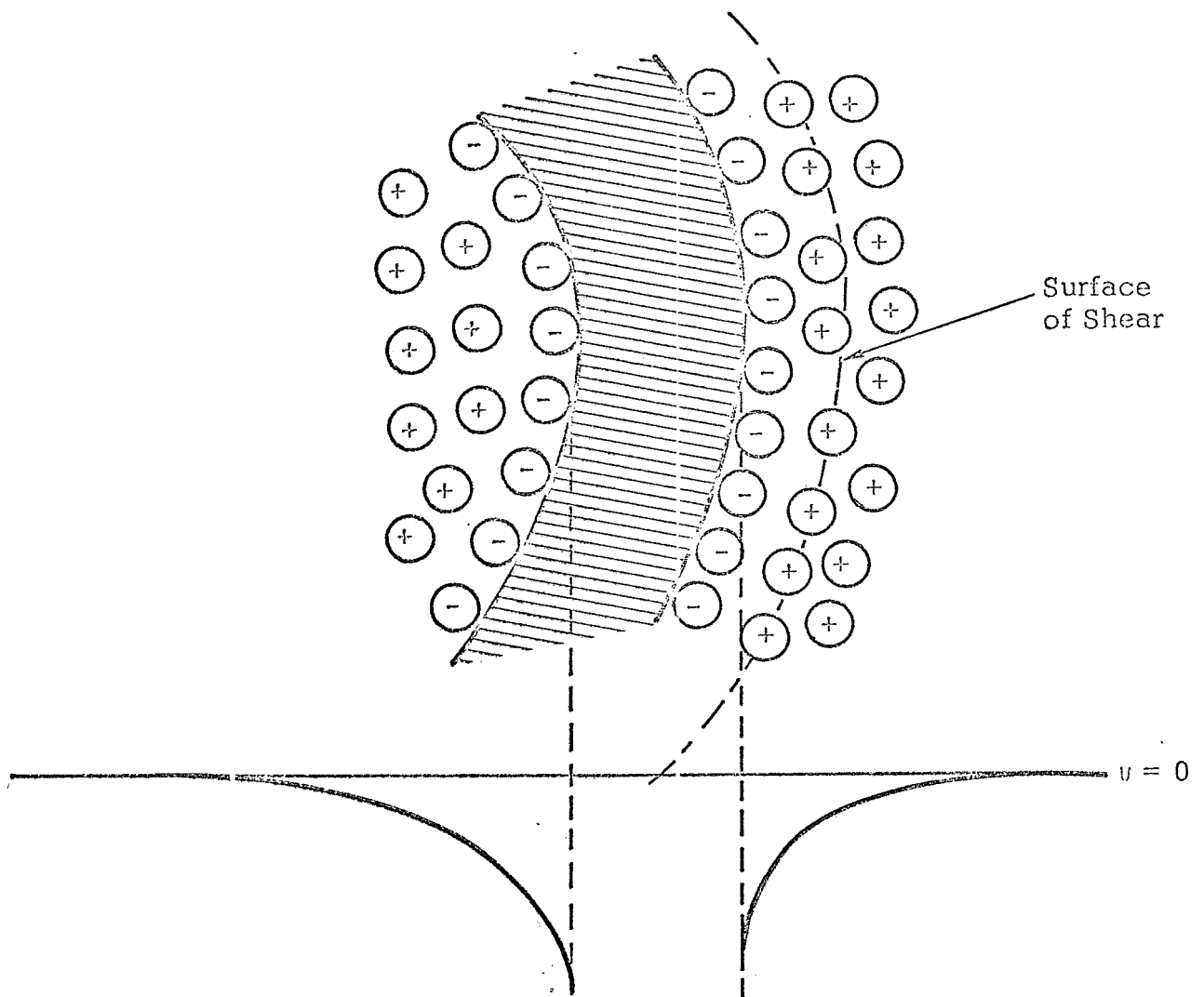


Figure 1-9. The cancellation of surface charge potentials. Both sides of the cell membrane (including the cell wall) have an excess of negatively charged dissociable groups at high pH values when amino groups are not charged.

heating, exposure to formaldehyde fumes, to ethylene oxide and to chlorine. To insure that the above exposure treatments rendered the spores nonviable, samples of the suspensions were cultured on both Fluid Thioglycollate Media and Trypticase Soy. It was found that the treatments applied did induce sterility. The killed spore suspensions were used in the same way that the viable suspensions were used to determine what differences if any could be detected.

3. Results

The apparent isoelectric point of untreated B. subtilis spores was determined by microelectrophoresis to be 2.5. At pH ranges above and below 2.5 there were no reproducible differences between the viable and nonviable cells (Figures I-10 through I-14).

4. Conclusions

Electrophoresis of living and dead cell preparations did not show differences of sufficient order of magnitude to provide a useful means for differentiating between viable and nonviable cells. In order to provide a more sensitive means for the measurement of electrophoretic mobility as a function of pH it would be necessary to refine the technique. However, based upon the experiments performed upon the suspensions of B. subtilis, the order of magnitude of difference between viable and nonviable cells is so small that it is doubtful that even with highly refined apparatus that this method would show promise.

F. AUTORADIOGRAPHY

1. Introduction

A cell is metabolizing but not reproducing may in theory accumulate radioisotope-labelled metabolites and thereby make itself radioactive. By use of the technique of autoradiography (Joftes 1963) these cells which have accumulated radioactive tracers may be visualized on a photographic plate (Figure I-15).

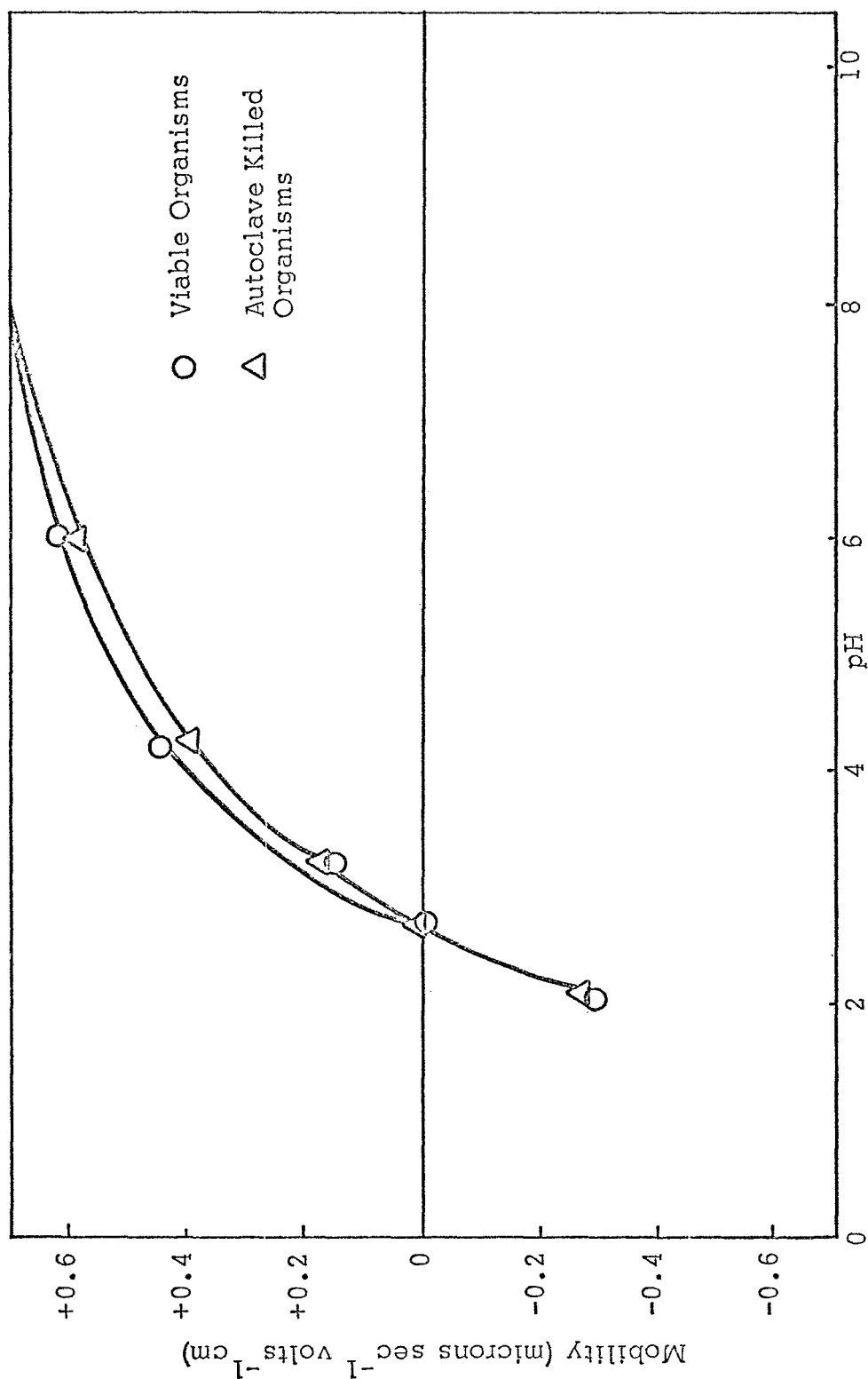


Figure I-10. The Effect of pH on the Mobility of Viable and Autoclave Killed Spores of B. subtilis var. niger

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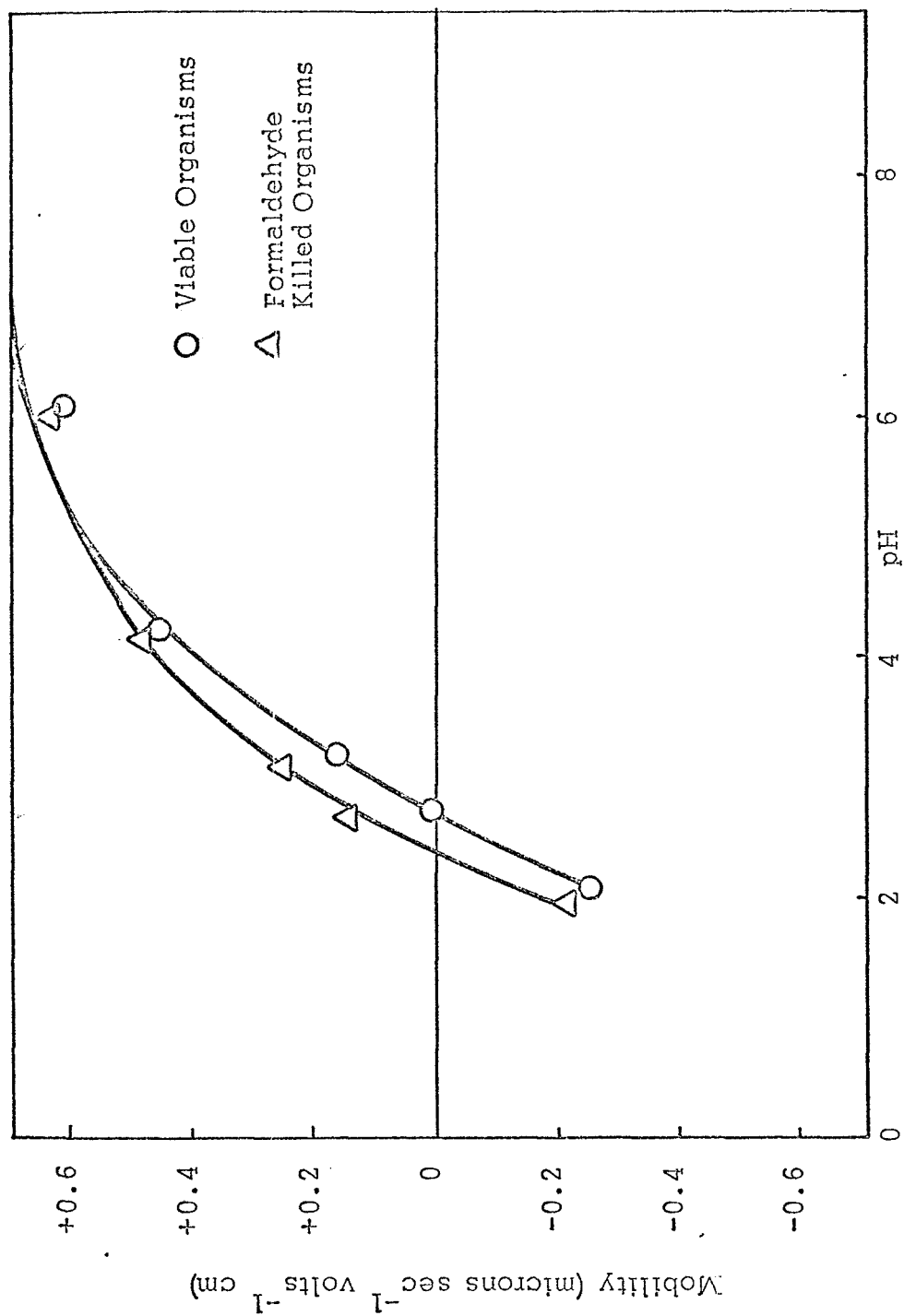


Figure I-11. The Effect of pH on the Mobility of Viable and Formaldehyde Killed Spores of B. subtilis var. niger

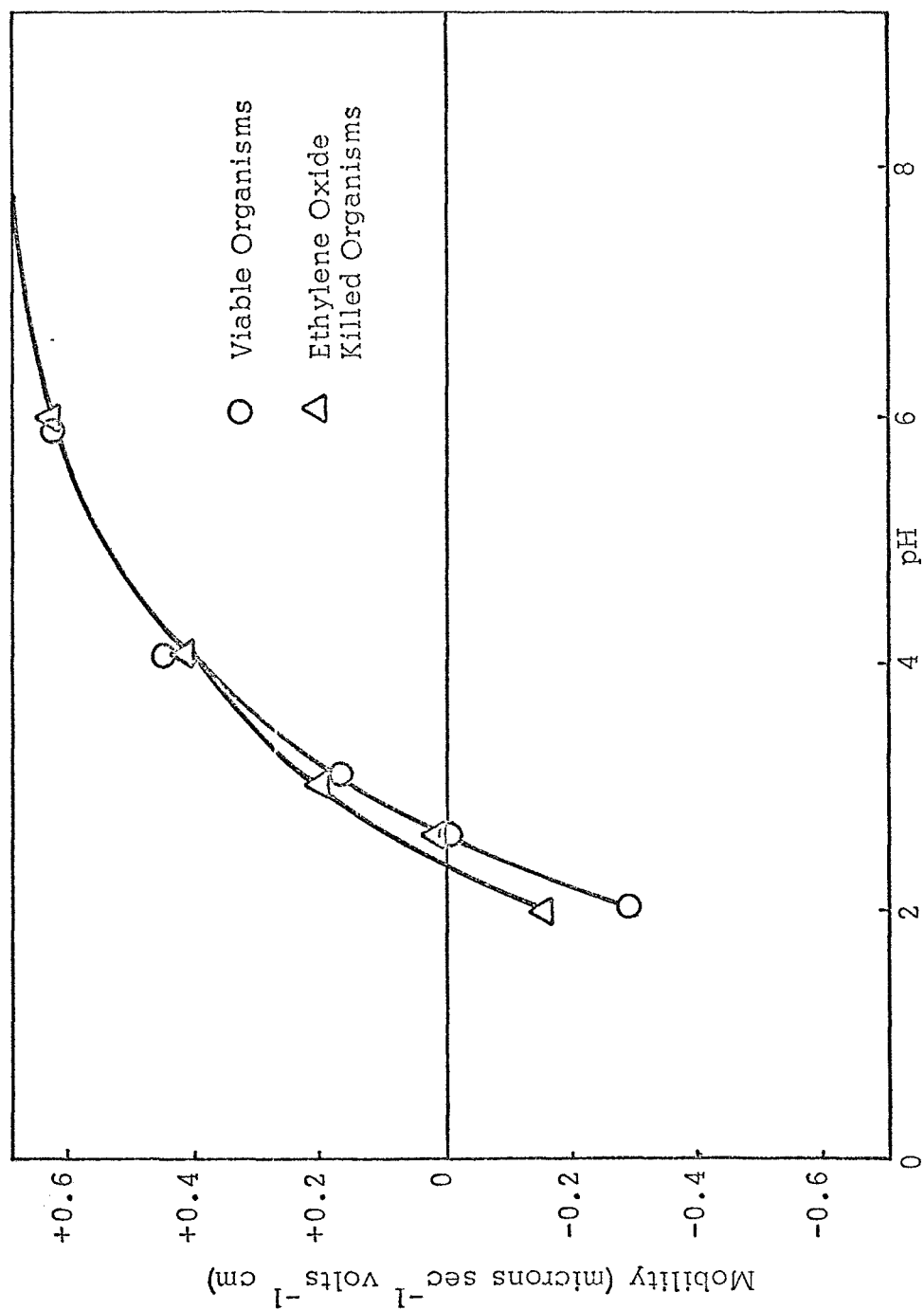


Figure I-12. The Effect of pH on the Mobility of Viable and Ethylene Oxide Killed Spores of B. subtilis var. niger

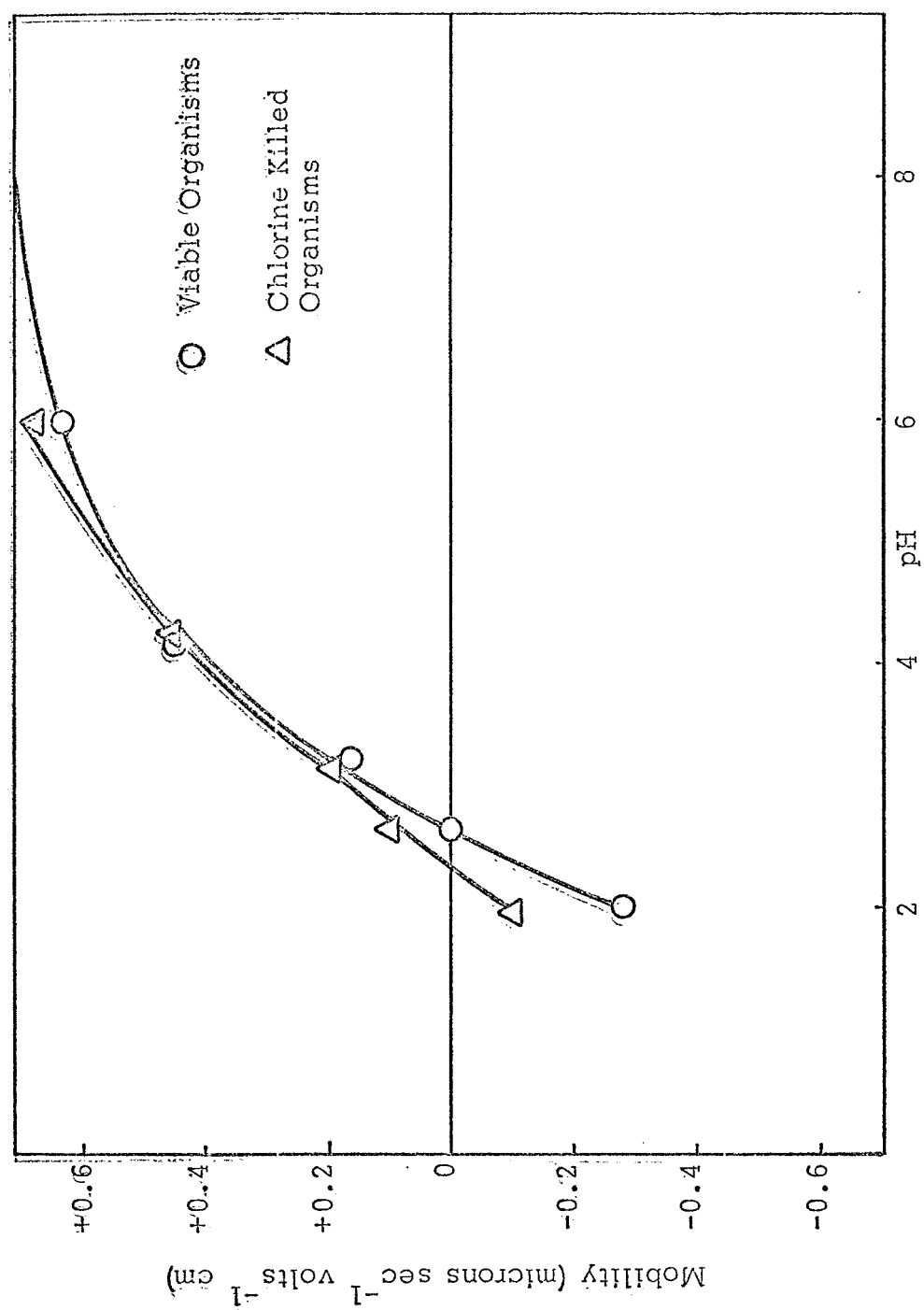


Figure I-13. The Effect of pH on the Mobility of Viable and Chlorine Killed Spores of B. subtilis var. niger

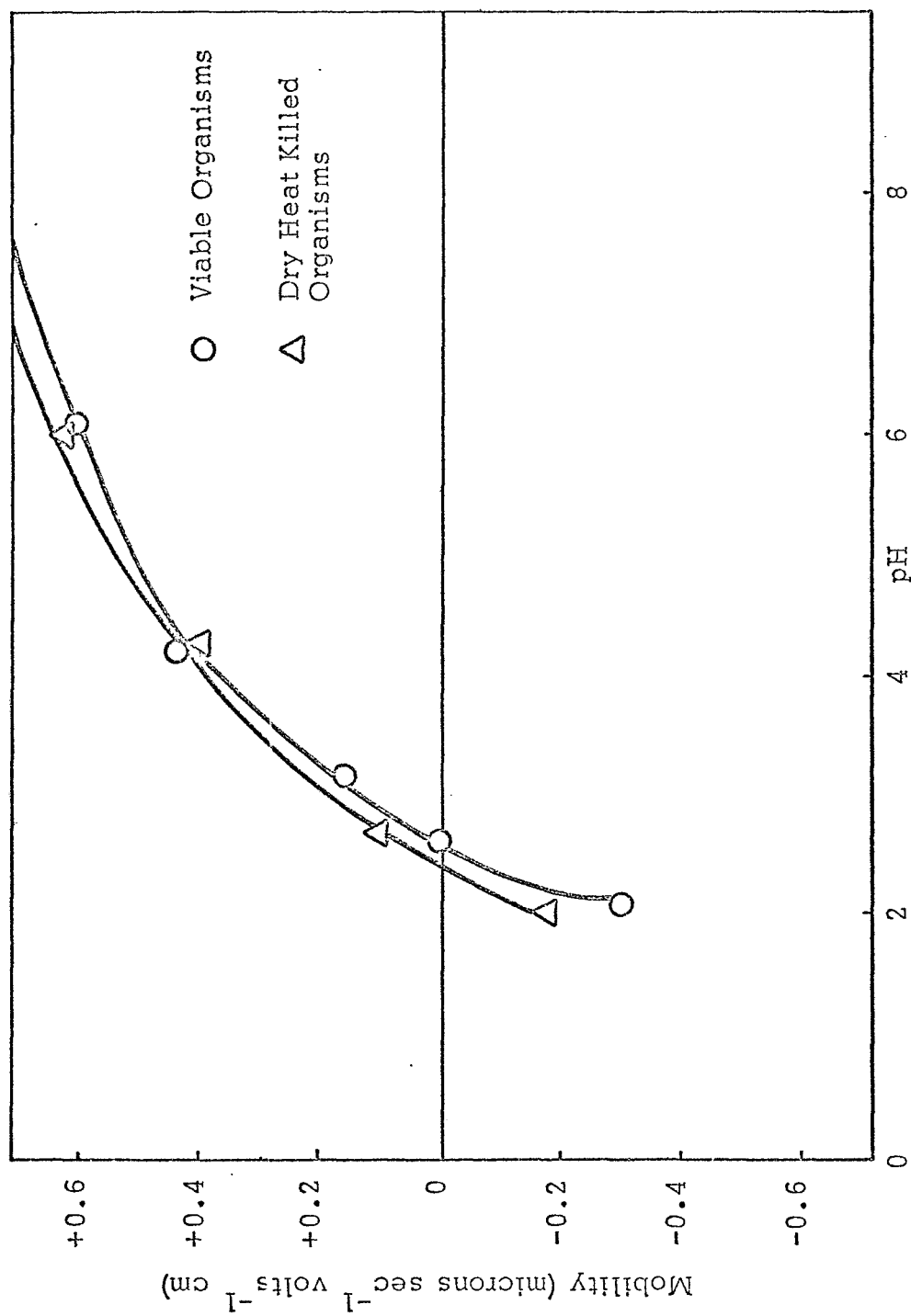


Figure I-14. The Effect of pH on the Mobility of Viable and Dry Heat Killed Spores of B. subtilis var. niger

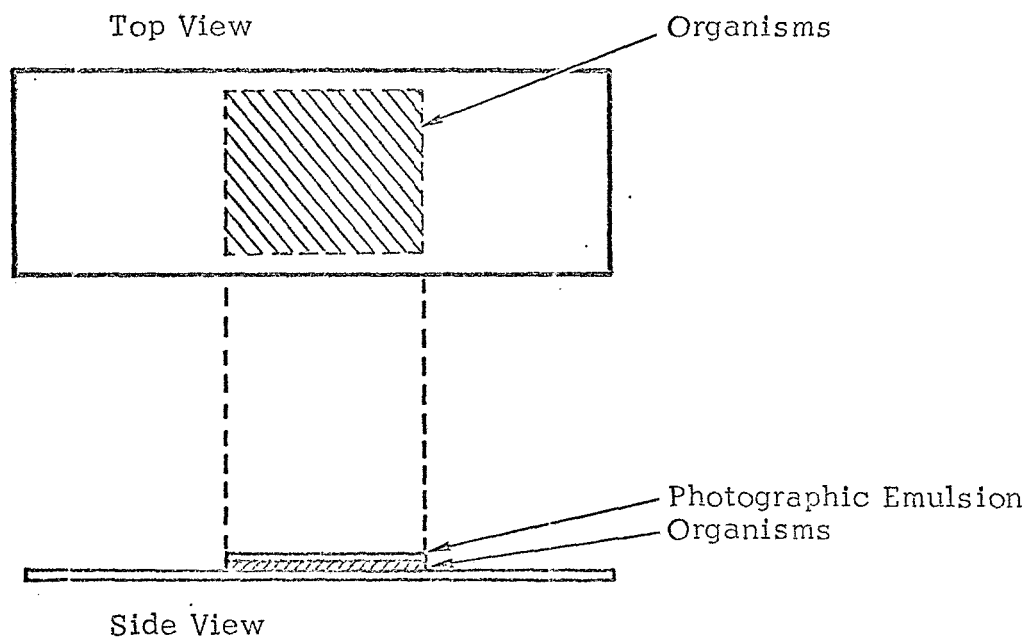


Figure I-15. Radio Autographs

The principle of this method depends upon placing the organism rendered radioactive into intimate contact with a photographic emulsion which is sensitive to very weak beta radiation. The weak beta rays then strike silver crystals causing opaque "squiggles" at the site which is struck by the ray. The pattern of opaque silver on the emulsion after photographic development ideally should be similar to that of the shape of the microorganism. If the sample is stained, the silver grain should be observed within the boundary of the cell.

2. Methods

a. Organisms

Samples of viable spores of B. subtilis were killed by dry heat, autoclaving, exposure to ethylene oxide to ultraviolet irradiation, and to chlorine.

(1) Viable Spores. The viable spores were suspended in water to give a concentration of 10^8 cells per centrifuge tube (100 mg spores). The viable count of these spores was found to be 7.7×10^{10} viable spores per 100 mg.

(2) Heat Killed Spores. 100 mg of lyophilized spores (7.7×10^{10} organisms per centrifuge tube) were exposed to dry heat at 180°C for a period of 2 hours. 20 ml of sterile distilled water was added to the centrifuge tube.

(3) Ethylene Oxide Killed Spores. 7.7×10^{10} spores were exposed to ethylene oxide for a period of 8 hours at 40% relative humidity. They were suspended in 20 ml sterile distilled water.

(4) Autoclave Killed Spores. 7.7×10^{10} spores were autoclaved for 20 minutes at 15 lbs. pressure in 20 ml distilled water.

(5) Ultraviolet Killed Spores. 7.7×10^{10} spores distributed in a thin layer on a Petri dish were exposed in a sterile enclosure to an ultraviolet Sterilamp for a period of 4 hours at a distance of 6 inches.

(6) Chlorine Killed Spores. 7.7×10^{10} lyophilized spores were exposed to dry chlorine gas for a period of 2 hours. The organisms were then suspended in 20 ml of sterile distilled water.

G. DISCUSSION OF DETECTION METHODS

1. Electrophoresis

The nonculturing techniques studied did not provide a reliable means for distinguishing between living and dead cells. The electrophoretic behavior and staining characteristics are dependent upon charge distribution and surface characteristics of the cell walls of microorganisms. Figure I-7 shows the types of binding sites on cell walls. Dye molecules which possess electrical charges opposite to those present on the cell walls of microorganisms may lend selectivity to such organisms.

Electrophoretic methods for the separation of charged particles including microorganisms provide a means for separating such particles and organisms according to their electrophoretic mobility. In the studies conducted in which viable organisms were contrasted with nonviable organisms, the differences in electrophoretic mobility were extremely low and in some cases nonexistent. Thus, under the conditions of the experiments conducted it is clear that electrophoresis does not provide a reliable means of differentiating between viable and nonviable organisms. It is also apparent that in a complex mixture of solids particles and microorganisms that solid particles which possess a similar electrical charge distribution and may have the same or similar electrophoretic mobility as microorganisms and thus obscure attempts to differentiate microorganisms from other solid debris.

2. Staining

While it is true that the binding or lack of binding of dyes to cell walls of microorganisms is not altered in a detectable way in nonviable as contrasted to viable microorganisms, the detection of organisms in either category is often helpful in assessing contamination levels. The presence of artifacts in stained samples of solids decrease the effectiveness of staining methods for the detection of viable or nonviable microorganisms.

The use of fluorescent staining techniques offers advantages over light microscopy staining techniques. The microscopic field background may

be so adjusted as to be essentially black. Microorganisms (living and dead) appear as brightly colored fluorescent structures against this background. Thus it is possible to microscopically scan since detection of a bright fluorescent object against a black background involves less fatigue and greater sensitivity since contrasting backgrounds do not appear when this method is ideally applied. Unfortunately, it is not possible to reliably differentiate between viable and nonviable organisms through the use of this method even under optimal circumstances since the differences between viable and nonviable cells involve differences in intensity of fluorescence.

3. Autoradiography

Autoradiography did not prove to be an effective method for the detection of microorganisms either viable or nonviable. The requirements inherent in the use of autoradiography are: (1) the cell must be permeable to the radioactive compound used and should retain the compound when washed, (2) the solid must be sufficiently uniform in texture such that less than 2.0 microns distance exists between organisms containing the radioactive label and the photographic emulsion, (3) that the solid does not nonspecifically bind the radioactive compound.

In the experiments conducted using radioautography, the prolonged time interval required even with large populations of spores would indicate that this method is relatively insensitive and time consuming. In addition if this method were to be applied to solids it would be almost impossible to pulverize solids uniformly without destroying the structural integrity of the organisms. Since the examination of the radioautograph is dependent upon the structural outlines of the cell, it is obvious that this method would be tedious and would lend itself to the detection of artifacts in addition to being insensitive.

4. Electron Spin Resonance

Electron spin resonance provides a means of detecting atoms or molecules possessing unpaired electrons (free radicals). Living systems contain enzymes which carry out biological oxidations and reductions which at some stage of electron transfer produce free radicals which may

be detected by the use of ESR resonance. Thus, it would appear that if only living systems contain free radicals, ESR spectroscopy would provide an elegant nondestructive method for the detection of viable microorganisms in solid materials. Unfortunately, a large number of organic and inorganic substances exist which contain free radicals and which cannot be differentiated from the free radicals generated in living systems. Since the presence of variable quantities of free radicals of differing species may be demonstrated in many types of materials it does not appear that this tool would be useful for the detection of microbial contamination of solid materials.

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PHASE II. Freeing Microorganisms from Encasement

Certain representative solid materials were subjected to pulverization by drilling, ball mill, Waring blender, and mortar and pestle. The most useful of these methods of pulverization was found to be drilling. The viability of the microorganisms was established by culture methods. Attempts were likewise made to dissolve solids thereby freeing the microorganisms from the solid material. Very little difference was noticed whether organisms were freed from encasement by pulverization or by dissolving in a suitable solvent. It was established that certain of the solvents exerted toxic effects upon the microorganisms. The use of drilling without attempting to dissolve the solid was the pulverization procedure used in all subsequent experiments in Phases III and IV. The recoveries of B. subtilis var. niger were disappointingly low. In a typical experiment the solid contained $10^9 - 10^{10}$ microorganisms per gram. When 0.1 gram of the solid material was pulverized, only approximately 10^3 viable microorganisms could be demonstrated rather than an expected total count of $10^8 - 10^9$ microorganisms.

II. PHASE II. FREEING MICROORGANISMS FROM ENCASEMENT

A. INTRODUCTION

1. Statement of the Problem

Microorganisms may populate solids in significant numbers and yet be undetectable because the methods used for freeing them from encasement may cause a high percentage of them to be so damaged as to not survive the treatment. The particle size distribution of solids which may be optimal for small spores and bacteria, may not be suitable for application to large mycelial cells of fungi (Figure II-1). Some solids, because of their physical-chemical nature may be solubilized. Microorganisms contained within such solids may be detected by cultural methods if the solvent and/or other environmental efforts have not caused their destruction. The major problem in dissolving the solid is the rather small number of solids which may be solubilized without using combinations of heat, pressure and solvents which destroy microorganisms.

Plastics represent one of the largest categories of solids that may be contaminated with microorganisms. Information regarding the range of possible solvents and plasticizers which may be used for particular types of plastics have been discussed by Doolittle (1954).

In general, plastics which crosslink or which are thermosetting like epoxys are insoluble. Polyethylene is an exception to the class of thermosetting polymers. This plastic material will dissolve in xylene at temperatures over 50°C. Polystyrene dissolves in a variety of aromatic hydrocarbons at 25°C. These solvents are, however, toxic to most microorganisms (Snell 1954).

2. Theoretical Approaches

Pulverization represents another method of freeing microorganisms from encasement in solids. A wide variety of methods may be applied to reduce a solid to a state of subdivision satisfactory for cultural examination

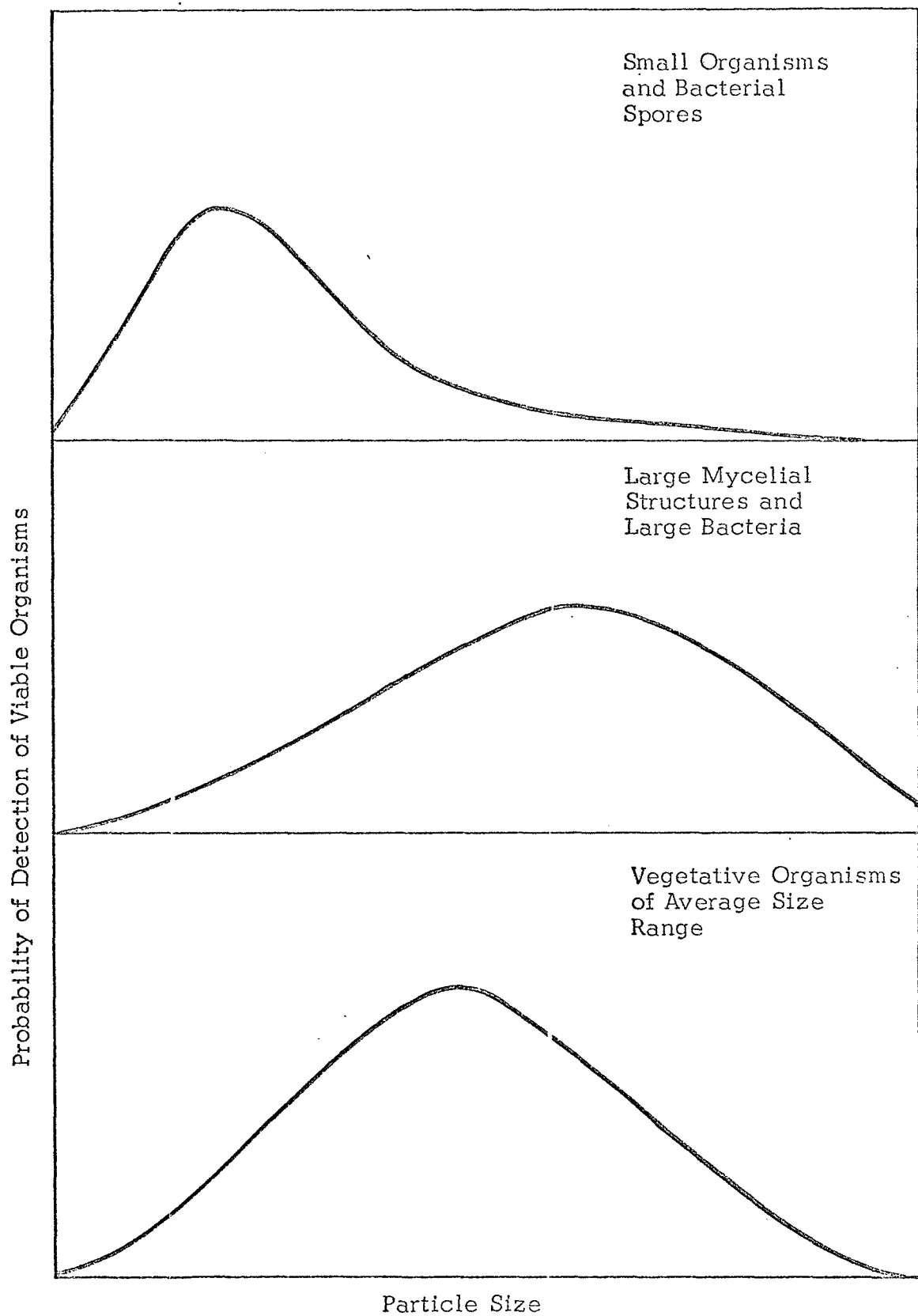


Figure II-1. Relationship between the Probability of the Detection of Viable Microorganisms in Solids and the Size of Particles

for microorganisms, The mortar and pestle has been used to pulverize various types of solids used as spacecraft components (Phillips 1960; Hoffman 1960). The latter technique relies upon crushing and shearing action. Morelli (1962) applied abrasion methods to pulverize a resin containing microorganisms added as inocula. A modification of this general method was also used by Portner (1962, 1963). Small pieces of solids may be pulverized by the use of crushing using a ball mill type of action. Mickle (1948) used this method for the disruption of materials. Ross (1963) describes a small efficient type of ball mill.

The use of the following methods of pulverization were investigated in Phase II for culturally recovering microorganisms added as inoculum:

- (1) mortar and pestle
- (2) ball mill
- (3) Dremel drill
- (4) Waring blender

Attempts were also made to solubilize the pulverized material prior to culturing.

B. METHODS

1. Organisms

Spores of B. subtilis were obtained as dry lyophilized spores from Ft. Detrick, Md. These spores were stored until needed at 5°C. When required for use as inocula, they were suspended in dilution bottles and assayed in Trypticase Soy Agar plates by decimal dilution. The number of culturally viable organism/gm was accurately established when inocula were prepared for addition to solids.

2. Materials

Paraplast is a hydrophobic material which is relatively soft, flexible and of moderate impact strength. It is easily sliced, producing large chips.

Parlodion is a cellulose nitrate derivative which has a hardness in excess of that measurable on the Shore A scale. It has high flexural and impact strengths. Its hardness makes it a difficult material to slice, or pulverize in a mortar and pestle; however, it may be drilled or pulverized in a ball mill and with considerable difficulty in a Waring blender.

Plaster of paris is a moderately hard, hydrophilic material possessing low flexural and impact strengths. It may be easily pulverized by all methods used in Phase II except the microtome. Attempts to slice this material with the latter instrument consistently lead to powdering and crumbling.

Eccocoat IC2 is a polyurethane coating material moderately hard with high flexural and impact strengths. The manufacturer describes it to be funginert, indicating that it is possibly toxic to some microorganisms.

3. Inoculation of Solids

The specimens were prepared with inocula of B. subtilis at levels indicated in Tables II-1 through II-4. No attempt was made to sterilize the materials before inoculating them. Because of the presence of B. subtilis spores in the laboratory where the work was performed the chance of accidental contamination could not be overlooked. The aluminum molds (Figure II-2) which were used in casting of the inoculated solids were autoclaved before use and sterile technique was used in the casting of the specimens. All materials were cured at room temperature. The inocula were added to the materials and mixed thoroughly just before the solid showed evidence of hardening. The inoculated solids were cured at room temperatures and maintained at room temperature until assayed.

4. Pulverization of Solids

With all of the methods of pulverization except the Dremel drill, the organism was cooled to liquid nitrogen temperature just before pulverization. In the case where drilling was used, the sample was drilled while immersed in autoclave sterilized culture media for a period of 1/2 minute.

The mortar and pestle were used inside a decontaminated polyethylene bag. The solid was pulverized for two minutes and transferred to sterile Trypticase Soy Agar plates and cultured.

The blender vessel (one quart capacity) was autoclaved. The solid was added and pulverized for one minute at top speed. It was rinsed from the blender bowl and transferred to the Trypticase Agar plates.

5. Cultural Assay

A limit exists to the size of population of any particular species of microorganism which can be assayed on a single agar surface. As colonies become more numerous, the frequency of two or more cells lying so closely together that their colonies overlap increases. The colonies which develop early deplete the agar of nutrient and thereby suppress the development of visible colonies from cells which start proliferation later.

Trypticase soy agar plates were chosen for the assay medium because of their ability to produce a high degree of germination and colony development in spores of B. subtilis. They also permit easy quantitative measurement of the proportion of the inoculum recovered. The program at Phase II was not really addressed to precise measurement of recovery percentages in the easy cases but was addressed to the development of a method of promise for measuring the internal populations of spores in materials difficult to test for sterility.

6. Determination of Particle Size

Each of the various solids was encased in the aluminum molds in the same manner indicated in Figure II-2. The samples were subjected to drilling while immersed in culture media. The particles were removed from solution by centrifugation at $2000 \times g$ for 1 hour in order to sediment the finest particle size present. The sediment was resuspended in a small volume of distilled water and examined microscopically. 500 particles were counted and the results are shown in Figures II-3 through II-6.

The samples were also subjected to pulverization using the ball mill, mortar and pestle, and Waring blender. With the exception of plaster of

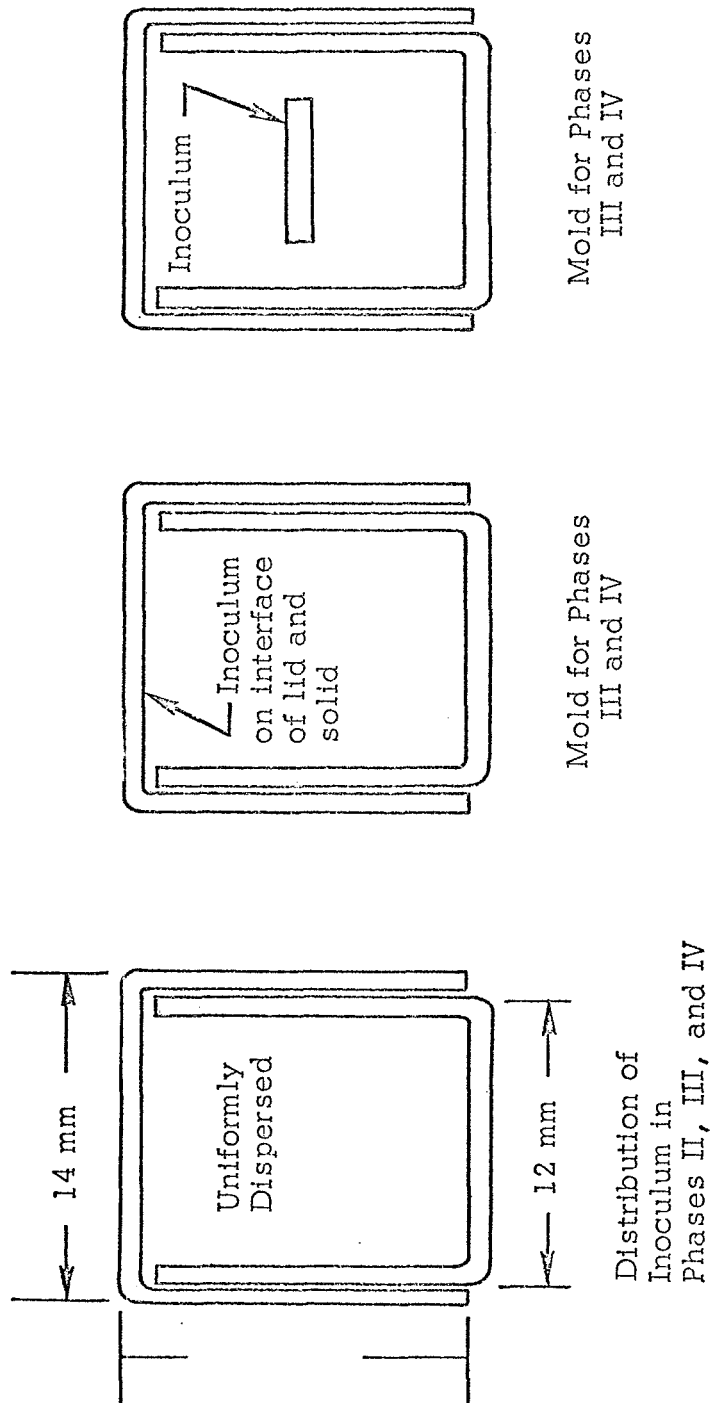


Figure II-2. Locations in Inocula in the Solid Materials

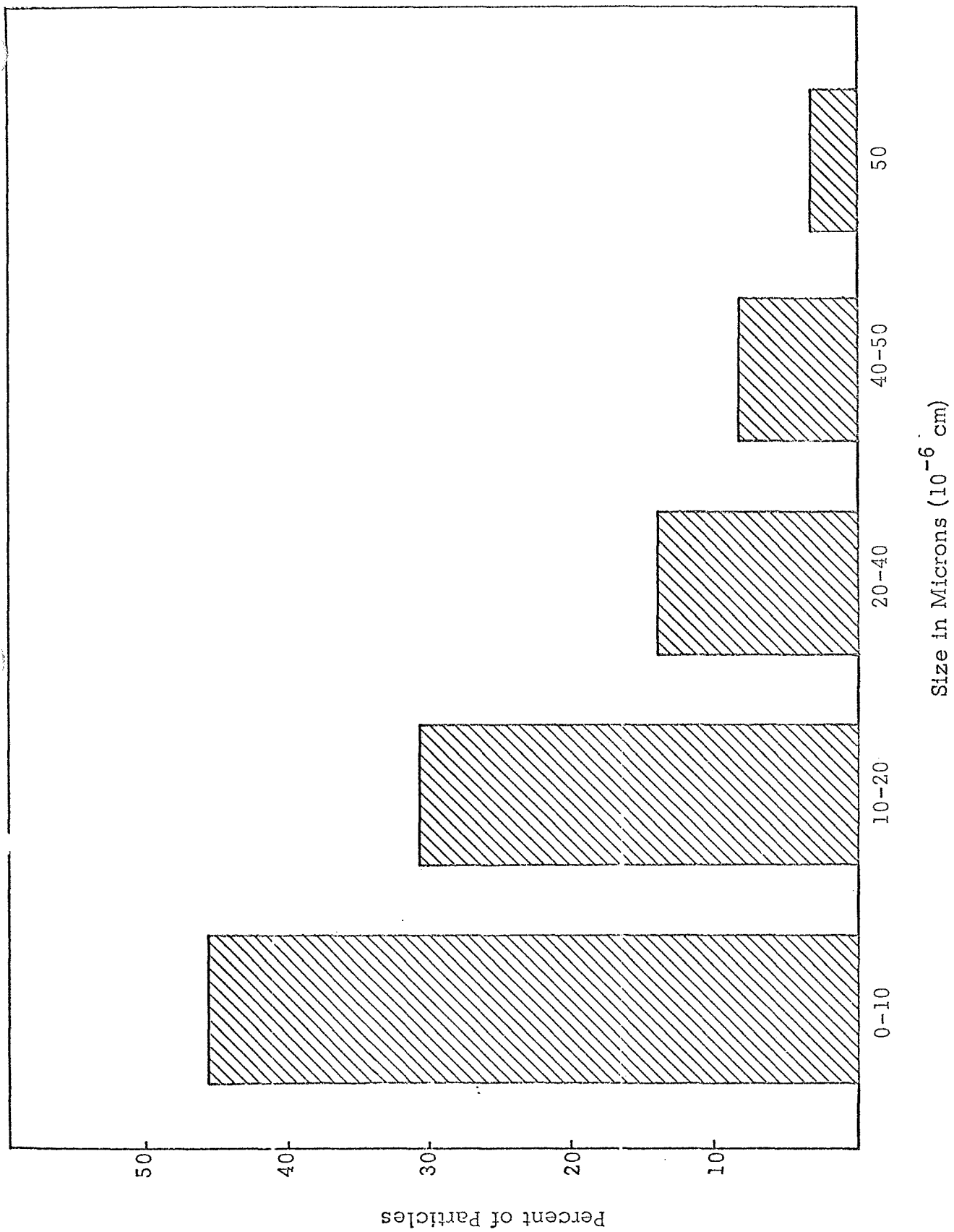


Figure II-3. Particle Size Distribution of Plaster of Paris Pulverized by Drilling

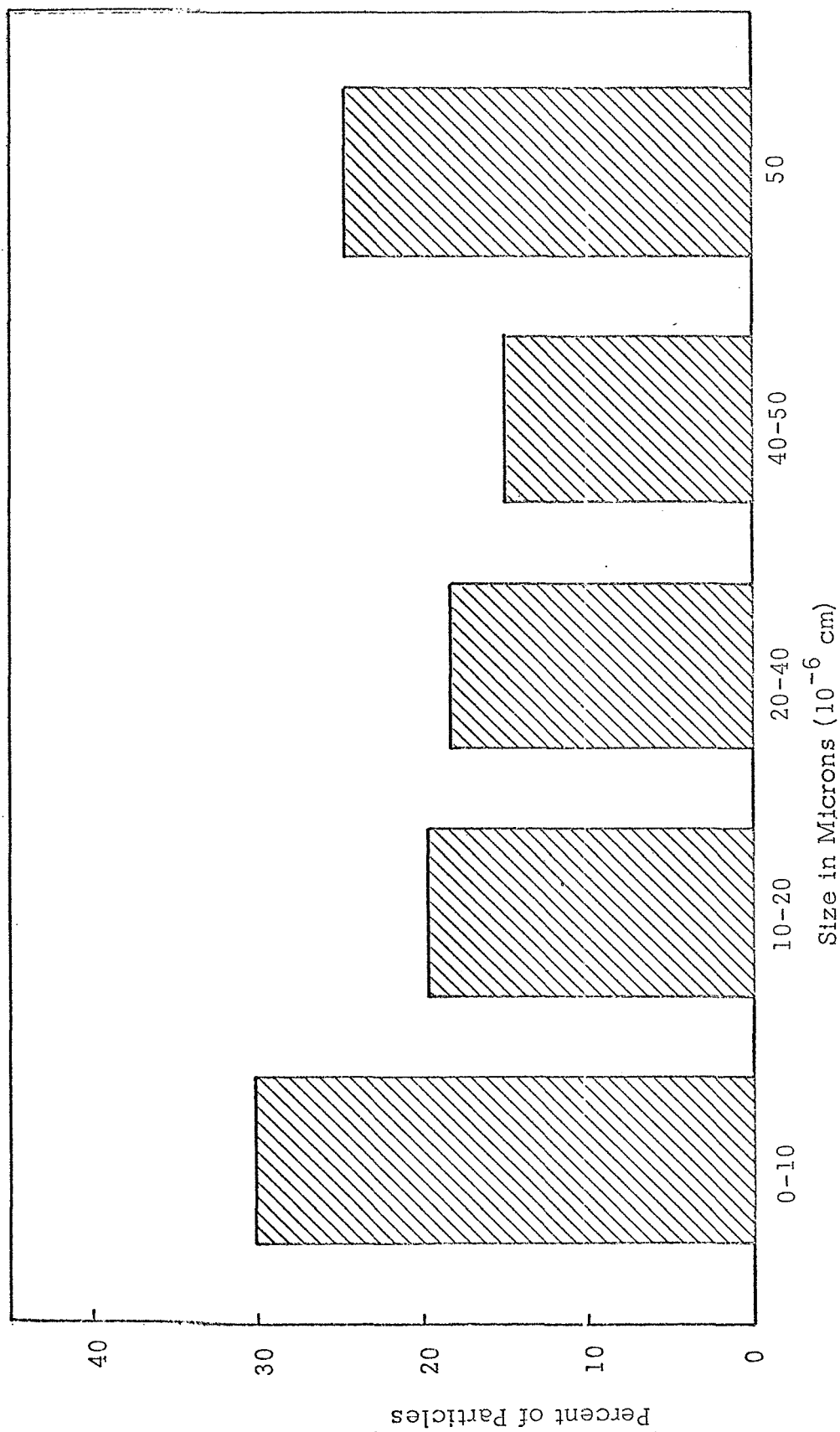


Figure II-4. Particle Size Distribution of Parlodion Pulverized by Drilling

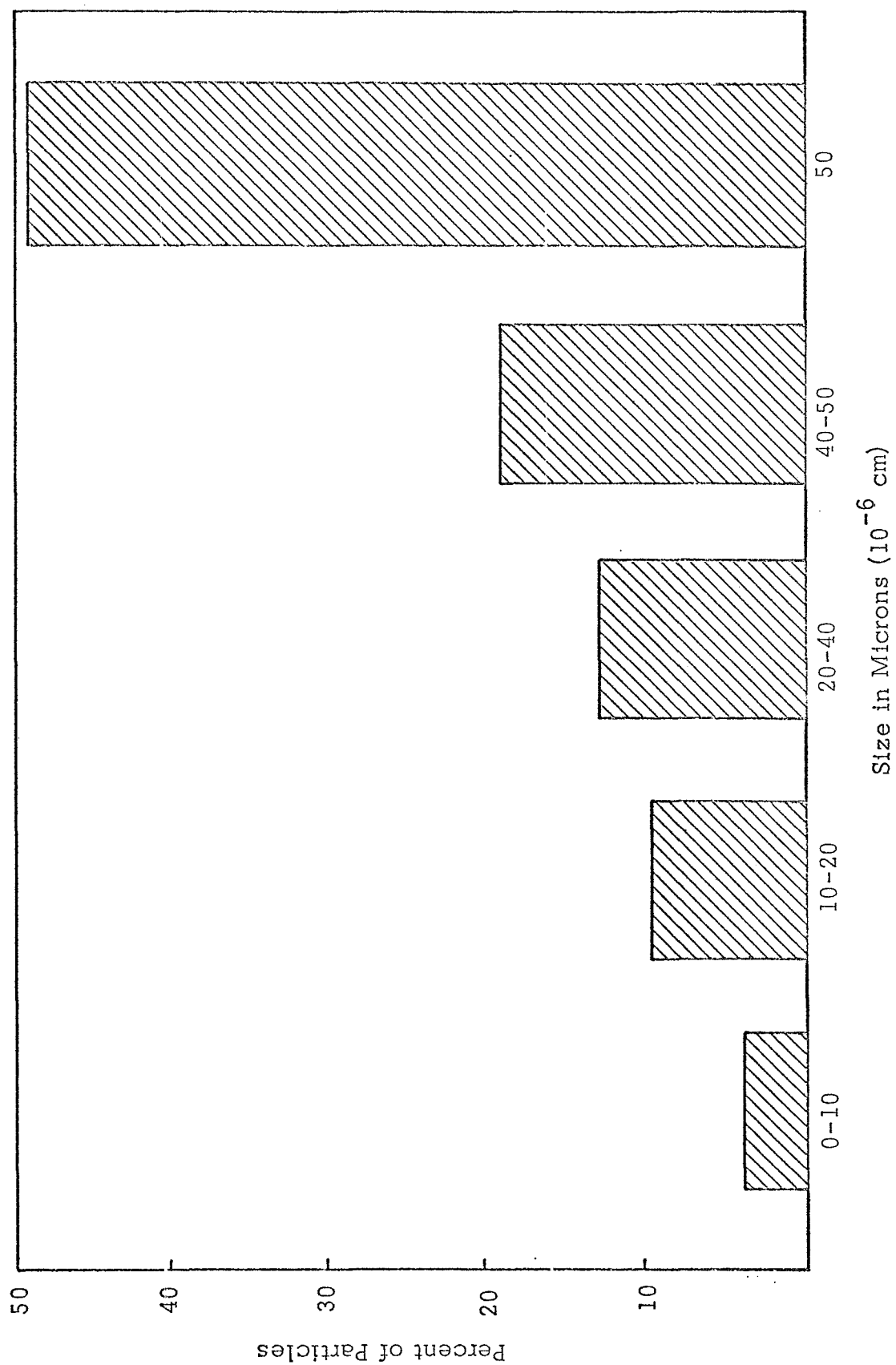


Figure II-5. Particle Size Distribution of Stycast 2651 Pulverized by Drilling

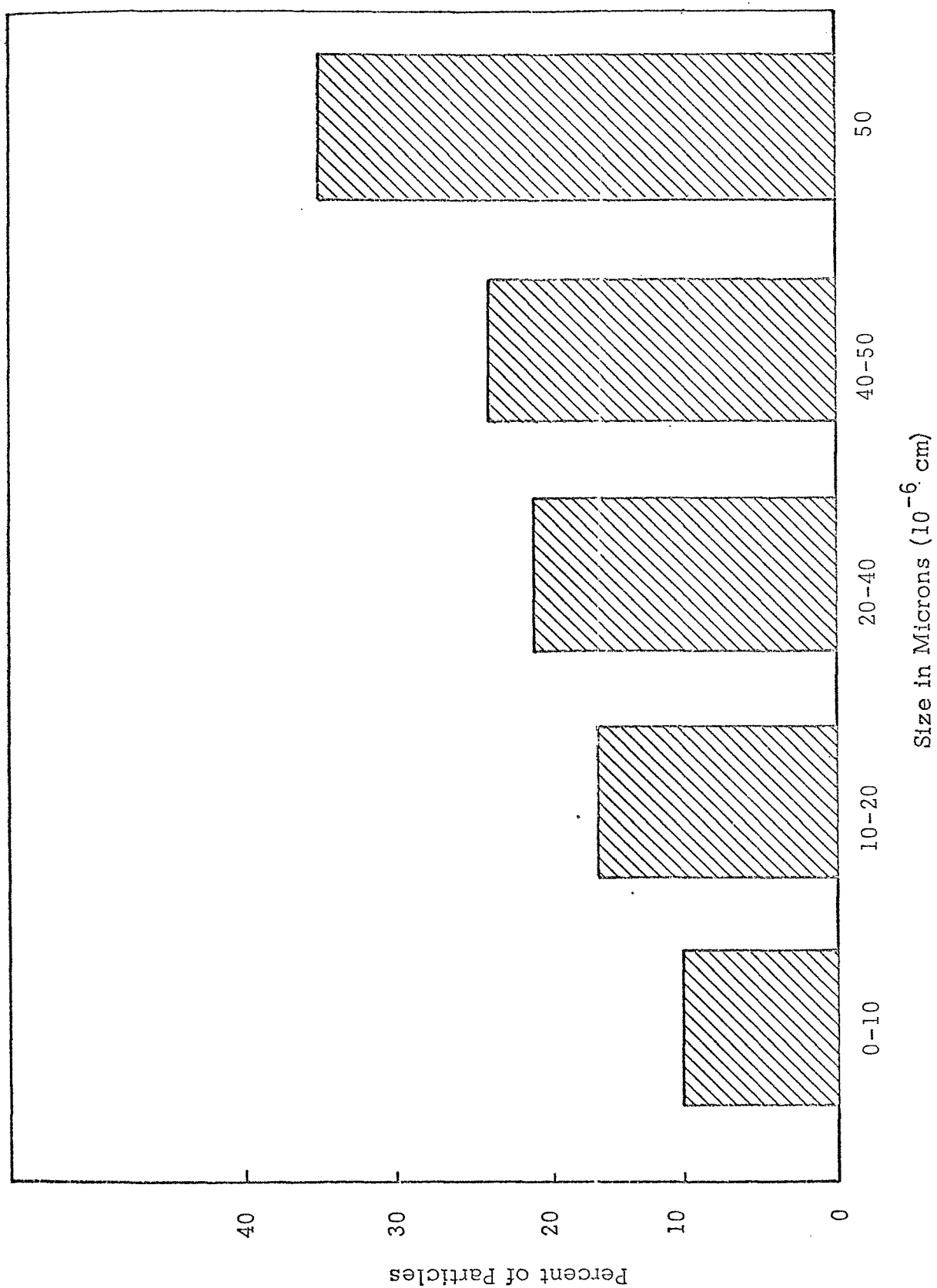


Figure II-6. Particle Size Distribution of Piccotex 120 Pulverized by Drilling

paris, none of the above methods produced satisfactory pulverization. The plaster of paris could be fragmented to particles less than 10 microns; however, the range of distribution of particle sizes varied considerably. The Waring blender pulverized the parlodion particles after preliminary freezing with liquid nitrogen. Unfortunately, however, as the particle sizes were reduced below 25 microns, the resultant aerosol ignited and exploded.

For paraplax (wax) one ml of the acetone suspension of its particles from Specimen No. 2 was diluted 10 to 1 in xylene and an aliquot of the resulting solution was used to inoculate the test agar.

One ml of the water suspension of parlodion particles was dried in an aluminum dish and the residue was dissolved in 10 ml of acetone. An aliquot of the resulting solution was placed on the test agar.

One ml of the acetone suspension of plaster of paris particles was diluted to 10 to 1 in 3% ethylene diamine tetraacetic acid (EDTA) solution. Again an aliquot of the resulting solution was assayed for viable spores.

One ml of the water suspension of Eccocoat IC2 particles was dried in an aluminum dish. The residue was dissolved in dimethyl formamide and the resulting solution was assayed for viable spores.

7. Experimental Design of Recovery Experiments

For each of the four materials and each of the pulverizing processes three inoculated specimens were prepared. These specimens were processed in the following way:

Specimen No.	Pulverized	Cultured Directly	Cultured After Dissolving Solid Material
1	yes	yes	no
2	yes	no	yes
3	no	no	yes

Specimen No. 1 indicated the overall recovery portion for the process and material. Specimen No. 2 (compared with No. 1) showed how many spores were freed when the particles of solid were dissolved away from them. Specimen No. 3 permitted measurement of the toxic and bacteriostatic effects of the solid material and the solvent on the inoculum.

C. RESULTS

Recovery of viable organisms from the solids tested indicated that the drill provided the most effective means of pulverization compatible with culturing methods. The mortar and pestle, blender, and ball mill allowed recovery of smaller number of B. subtilis (Tables II-1 through II-4).

Obviously, the solids from which microorganisms may be most easily recovered are paraplast and plaster of paris (Tables II-1 and II-3). Recovery from parlodion and Eccocoat IC2 were consistently more difficult than the former two solids (Tables II-1 through II-4).

TABLE II-1
EFFECT OF VARIOUS METHODS OF PULVERIZATION AND SOLUBILIZATION ON THE
RECOVERY OF BACILLUS SUBTILIS VAR. NIGER FROM PARAPLAST

Treatment of Sample	Inoculum (Organisms/ gm Solid)	Theoretical No. Colonies Based on Size of Inoculum	Actual No. of Colonies Observed			
			Drill	Ball Mill	Mortar and Pestle	Blender
1. Pulverized	2×10^8	10^8	6×10^3	5×10^2	5×10^3	2×10^3
2. Pulverized and Solubilized	2×10^8	10^8	4×10^3	3×10^3	5×10^3	8×10^3
3. Solubilized	2×10^8	10^8	$6, 5 \times 10^3$	--	--	--

TABLE II-2

EFFECT OF VARIOUS METHODS OF PULVERIZATION AND SOLUBILIZATION ON THE
RECOVERY OF BACILLUS SUBTILIS VAR. NIGER FROM PARLODION

Treatment of Sample	Inoculum (Organisms/ gm Solid)	Theoretical No. Colonies Based on Size of Inoculum	Actual No. of Colonies Observed			
			Drill	Ball Mill	Mortar and Pestle	Blender
1. Pulverized	$10^9/\text{gm}$	5×10^7	2×10^2	Less than 10	10^2	50
2. Pulverized and Dissolved	$10^9/\text{gm}$	5×10^7	10^3	Less than 10	30	30
3. Dissolved	$10^9/\text{gm}$	5×10^7	9×10^3	--	--	--

TABLE II-3

EFFECT OF VARIOUS METHODS OF PULVERIZATION AND SOLUBILIZATION ON THE
RECOVERY OF BACILLUS SUBTILIS VAR. NIGER FROM PLASTER OF PARIS

Treatment of Sample	Inoculum (Organisms/ gm Solid)	Theoretical No. Colonies Based on Size of Inoculum	Actual No. of Colonies Observed			
			Drill	Ball Mill	Mortar and Pestle	Blender
1. Pulverized	10^9	2×10^8	10^4	2×10^3	5×10^3	5×10^3
2. Pulverized and Dissolved	10^9	2×10^8	5×10^3	7×10^3	8×10^3	9×10^3
3. Dissolved	10^9	2×10^8	7×10^3	--	--	--

TABLE II-4

EFFECT OF VARIOUS METHODS OF PULVERIZATION AND SOLUBILIZATION ON THE
RECOVERY OF BACILLUS SUBTILIS VAR. NIGER FROM ECCOCOAT IC2

Treatment of Sample	Inoculum (Organisms/ gm/Solid)	Theoretical No. Colonies Based on Size of Inoculum	Actual No. of Colonies Observed			
			Drill	Ball Mill	Mortar and Pestle	Blender
1. Pulverized	10^9	2×10^8	5×10^2	Less than 10	5×10^2	Less than 10
2. Pulverized and Dissolved	10^9	2×10^8	5×10^2	Less than 10	Less than 10	10
3. Dissolved	10^9	2×10^8	8×10^3	--	--	--

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PHASE III. Demonstration of the Applicability of Drilling Techniques
for Recovery of Microorganisms in Solids

A variety of solid materials were inoculated at three different locations with spores of B. subtilis var. niger, Clostridium sporogenes, and Ulocladium. The distribution of the inocula included the following: uniformly distributed throughout applied to the interface between the solid and the aluminum lid, and at the interface of an aluminum disc and the solid material. The microorganisms were exposed from the solid materials by the use of drilling techniques. The recoveries of microorganisms in all cases were extremely low. When the inoculum was uniformly distributed throughout the solid, the recoveries were higher than when the inoculum was applied at either of the two interfaces.

The solubility of the various solids in a variety of solvents were studied in Phase III. The toxicity of various solvents was determined.

III. PHASE III . DEMONSTRATION OF THE APPLICABILITY OF DRILLING TECHNIQUES FOR RECOVERY OF MICROORGANISMS IN SOLIDS

A. INTRODUCTION

The objective of Phase III concerns the demonstration of the applicability of the best method (drilling) in Phase II to several solid materials of different physical properties. The solid materials investigated in this phase includes those with a wide range of physical and chemical characteristics. The solids are representative of those used as potting compounds, plastic structural components, dielectrics, and coatings.

B. METHODS

1. Preparation of Solid Materials

The solid materials investigated are listed in Tables III-1 through III-4. All materials were mixed with the catalyst in the recommended ratios and the inoculum of microorganisms was added to the material just prior to its showing evidence of solidifying. The dental cups which are composed of soft aluminum alloy were autoclaved prior to filling with the solid materials. The three locations of the inocula in relation to the dental crown containers used as receptacles are shown in Figure III-1. The procedures for culturing drilled specimens are shown on Table III-5. The isolator, specimen, and drilling assembly is shown in Figure III-1.

2. Sectioning of Propellant

The solid propellant was cast with the inoculum at Jet Propulsion Laboratory, cured, and submitted to Dynamic Science. This material was not treated in the same manner as the other solids, but was sliced with a blade-type microtome and subsequently cultured. The microtome procedure involved the use of an adjustable device which advanced the propellant to the blade cutting surface. Slices of 400 microns were

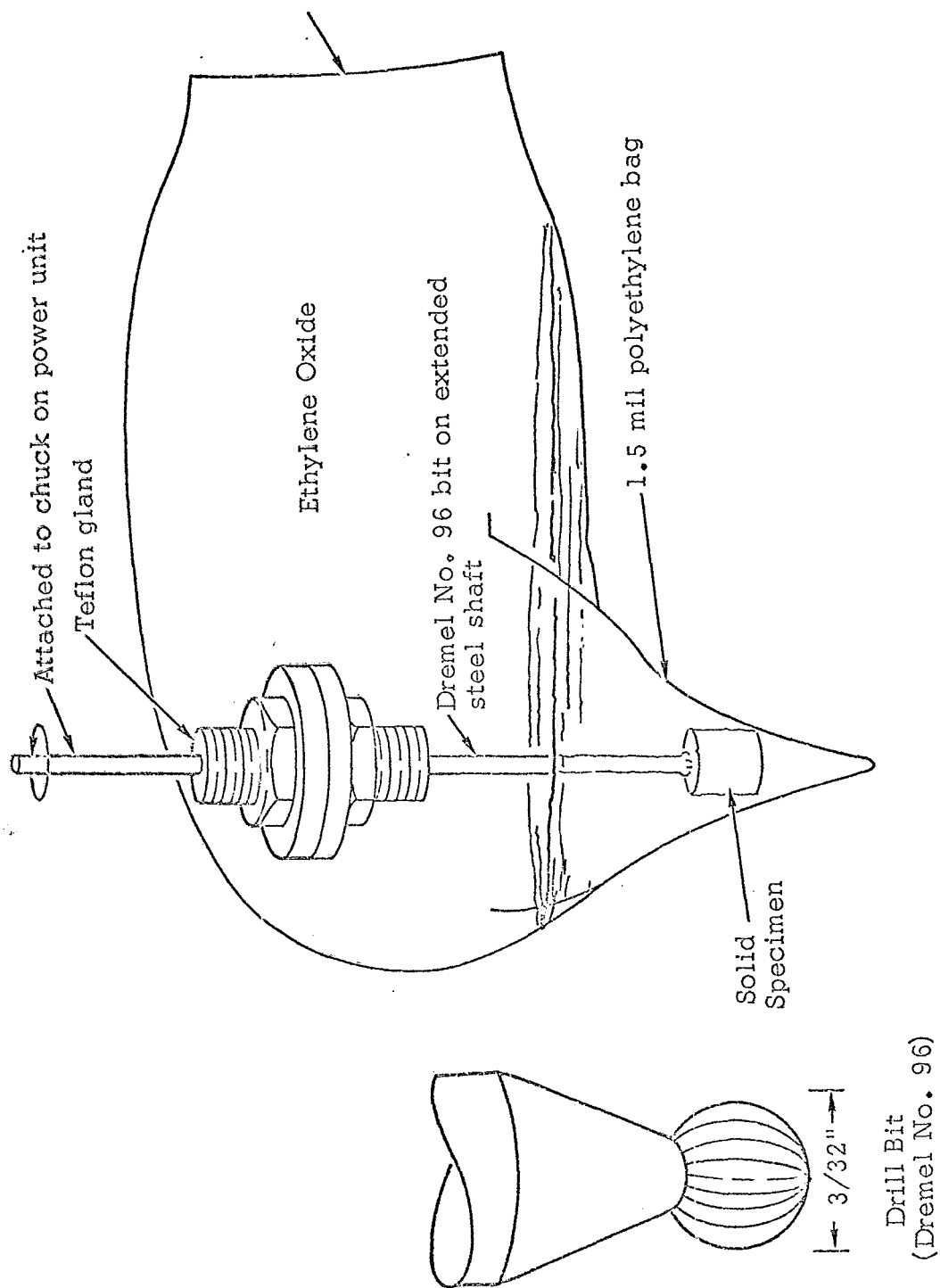


Figure III-1. Isolator, Specimen and Drilling Assembly

TABLE III-1
PROPERTIES OF VARIOUS SOLIDS STUDIED IN PHASES II AND III

Name of Principal Constituent	Common Use	Properties					
		Principal Constituent	Hardness, Shore A	Flex. Str. lb/in ²	Impact Str. ft lb/in of notch	Density g/ml	Water abs. %
Paraplast Parlodlon	dental casting many	paraffin cellulose nitrate	55 - 60				
Plaster of Paris	many	calcium sulfate	97				
Eccocoat IC2	coating	polyurethane	80				
Piccotex 120	casting resin	polystyrene	99				
Stycast 2850GT	casting resin	epoxy resin	90 - 95	13,300	0.24	2.0	0.1
RTV-40	potting compound	Silicone rubber	55			1.37	
Stycast 2651 MM	potting compound	epoxy polymer	92 - 97	12,000	0.2		0.15

TABLE III-2
PROPERTIES OF VARIOUS SOLIDS STUDIED IN PHASE III

Name of Principal Constituent	Common Use	Properties				Density g/ml	Water abs. %
		Principal Constituent	Hardness, Shore A	Flex. Str. lb/in ²	Impact Str. ft lb/in of notch		
Epon 901/B3	adhesive	epoxy polymer	92 -97				
Eccogel 1265	adhesive	epoxy gel	25			1.0	
Propellant	solid propellant	ammonium perchlorate and polyurethane	70				
RTV-40	foaming resin	silicone rubber foam	43 - 47				
Stycast 1090	foaming resin	epoxy foam	85 - 90	4200		0.78	0.1
CAT-A-LAC 443-1-500	coating coating		85 - 90 Rex				
Eccocoat IC2	coating	polyurethane	80				

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TABLE III-3

SOURCES OF MATERIAL

Material	Manufacturer or Supplier	Address
Epon 901/B-3 Eccogel 1265	Shell Chemical Company Emerson and Cuming, Inc.	Canton, Mass.
Propellant	Jet Propulsion Laboratory	Pasadena, Calif.
RTV-40 Stycast 1090	General Electric Company Silicone Products Department Emerson and Cuming, Inc.	Waterford, N. Y. Canton, Mass.
CAT-A-LAC 443-1-500 Eccocoat IC2	Finch Paint and Chemical Company Emerson and Cuming, Inc.	Torrance, Calif. Canton, Mass.

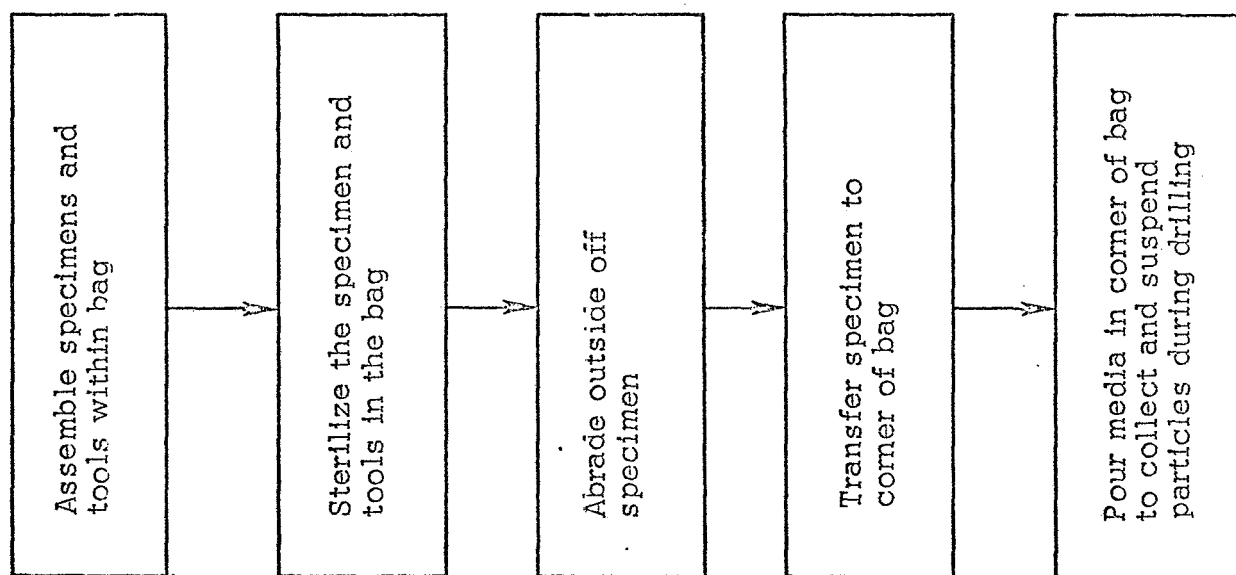
TABLE III-4

SOURCES OF MATERIAL

Material	Manufacturer or Supplier	Address
Paraplast	Brunswick Corporation	St. Louis, Mo.
Parlodion	Mallinkrodt Chemical Company	St. Louis, Mo.
Plaster of Paris	-----	-----
Eccocoat IC2	Emerson and Cuming, Inc.	Canton, Mass.
Piccotex 120	Pennsylvania Industrial Chemical Corporation	Philadelphia, Pa.
Stycast 2850GT	Emerson and Cuming, Inc.	Canton, Mass.
RTV-40	General Electric Company Silicone Products Department	Waterford, N. Y.
Stycast 2651MM	Emerson and Cuming, Inc.	Canton, Mass.

TABLE III-5

PROCEDURE FOR CULTURING DRILLED SPECIMENS



possible with the use of this device. Below this thickness, the slices fragmented and were discontinuous.

The sections were approximately $3 \times 10 \text{ cm}^2$. The core from which the propellant samples were taken was prepared by sterilizing the outer surfaces of the propellant with ethylene oxide vapor in polyethylene gas sterilant bags (Figure III-1). The ethylene oxide and water vapor concentrations were established through the use of gas chromatography. Attempts were made to pulverize propellant with the use of drilling techniques. Unfortunately, the cut fragments and chips adhered to each other and produced an agglomerated dense mass of material rather than dispersible particles.

Attempts were made to drill propellant which had previously been frozen in liquid nitrogen. Freezing, however, did not sufficiently alter the physical characteristics of the propellant such that pulverization in the absence of agglomeration was possible. Earlier work with propellant demonstrated that it may be pulverized by abrading with emery paper. However, appreciable loss would take place because the propellant would clog the abrasive on the paper and render it useless. Another method which was tried and found to have more promise than those previously used was abrading with a stickle back rasp.

The organisms studied in Phase III were B. subtilis, Clostridium sporogenes, Ulocladium, and Staphylococcus epidermidis. The organisms were prepared in mixed inocula, suspended in 0.01 ml of redistilled water and added to the ingredients of the solid materials just prior to the ingredients showing evidence of hardening. All materials in Phase III were inoculated as described except propellant. The total weight by Celite used for each level of mixed inoculum ranged between 2 and 3 grams. The Celite (Johns Mansville) was previously sterilized in a dry oven at 170°C for sixteen hours. The contents of the Celite organism inocula were added to 1000 gm propellant formula by personnel at Jet Propulsion Laboratory.

3. Preparation and Assay of Inoculum

The organisms studied in Phase III were B. subtilis, Clostridium sporogenes, Ulocladium, and Staphylococcus epidermidis.

a. Bacillus subtilis

Spores of this organism were obtained from Fort Detrick in the lyophilized state. These organisms were suspended in dilution vials and viability counts performed following decimal dilution by plate counting techniques within one day of their use as inocula in solids.

b. Clostridium sporogenes

Of the anaerobic spore formers, only Clostridium sporogenes (the National Canners Assoc. P.A. -3679) was sufficiently nonpathogenic to be convenient for the purpose of this program. The confined environment of cells encased within plastic solids suggests that spores might survive this type of anaerobic environment. The Clostridial spores were prepared by growing vegetative organisms in Brain Heart Infusion Broth (DIFCO). Calcium chloride was added as a supplement to the media at a final concentration of 1.0% to enhance sporulation. The organisms were harvested by centrifugation at approximately 1000 x g and the sediment was examined microscopically to insure that they were in the form of spores. The supernatant fluid was discarded and the spores were washed three times by alternately centrifuging and resuspending in sterile distilled water. The washed suspended cells were subjected to exposure to heat at 80°C for a period of 10 minutes to destroy surviving vegetative forms and to insure that only the spores survive. Following this heat shock step, the cells were stored at 5°C until required as an inoculum. In order to provide the inoculum at specific levels for JPL, the cells were assayed by decimal dilution in anaerobic deep agar tubes and by the use of Brewer Anaerobic Agar (BBL) plates.

c. Ulocladium

Spores of the fungus Ulocladium were originally isolated from dust obtained from the JPL assembly facilities. The fungus was identified as

Ulocladium by Dr. Emory G. Simmons, Head of the Mycology Laboratory of the U. S. Army Natick Laboratories. This fungal organism is most similar to Alternaria consortiale (Thum) Grove and Hughes. Although this organism resembles both Alternaria and Stemphylium, Ulocladium shows morphological differences.

The Ulocladium was grown on Sabouraud Agar medium (DIFCO). These organisms were harvested at the end of the first week of growth in distilled water and were subjected to sonication in an Acoustica Associates Model DR50 AH ultrasonic bath for a period of 20 minutes at 40 to 60 mca at a power output which produced cavitation. Following sonication the cells were centrifuged at 1000 x g for 10 minutes, the supernatant fluid discarded and resuspended in sterile distilled water. This procedure was repeated twice. The cells were then resuspended in sterile distilled water in a sterile dilution bottle and maintained at 5°C until required for use as an inoculum. At the time of submission of the organism to Jet Propulsion Laboratory, plate counts on Sabouraud Agar medium (DIFCO) were performed.

4. Measuring Solubility and Toxicity

The solubility of the various solids is indicated in Tables III-6, 7, and 8. The toxicity of the various solvents on the growth of B. subtilis is shown in Tables II-9 and 10.

C. RESULTS

Recovery of B. subtilis was obtained in certain instances whereas Clostridium sporogenes and Ulocladium were not recovered (Tables III-11 through 18). When the inoculum was distributed uniformly throughout the solid, recovery levels were superior to those obtained when the inoculum existed either at the interface of the lid and the solid or at the juncture of the aluminum disc and the solid. Rate of growth of spores of B. subtilis was depressed when grown in the presence of the various constituents of the solid plastics materials (Tables III-9 and 10). It is not surprising that organic solvents are generally toxic to spores of B. subtilis since some of these solvents are used to disrupt the cell membranes of microorganisms in biochemical enzyme studies.

TABLE III-6
SOLUBILITY OF SOLIDS IN VARIOUS SOLVENTS

S = Soluble; I = Insoluble

Solid Material	Solvent			
	Ethylene Diamine	Nitrilo Triethanol	Dimethyl Formamide	Carbon Disulfide
Eccocoat IC2	S	I	S	I
Cat-A-Lac	I	I	I	I
Stycast 1090	I	I	I	I
Epon 901	S	I	I	I
Stycast 2651	I	I	I	I
RTV-40	I	I	I	I
Stycast 2850	I	I	I	I

TABLE III-7

SOLUBILITY OF SOLIDS IN VARIOUS SOLVENTS

S = Soluble; I = Insoluble; C = Colloidal

Solid Material	Solvent			
	Glacial Acetic	Pyridine	Tetra- Hydrofuran	Turpentine
Eccocoat IC2	I	I	S	I
Cat-A-Lac	I	C	C	I
Stycast 1090	I	I	I	I
Eccogel 1265	I	S	I	I
Epon 901	C	S	I	I
Stycast 2651	I	I	I	I
RTV-40	I	I	I	I
Stycast 2850	I	I	I	I

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TABLE III-8

SOLUBILITY OF SOLIDS IN VARIOUS SOLVENTS

PS = Partial Soluble; I = Insoluble

Solid Material	Solvent			
	Toluene	Benzene	Methanol	Glycerin
Eccocoat IC2	PS	PS	I	I
Cat-A-Lac	I	I	I	I
Stycast 1090	I	I	I	I
Epon 901	I	I	I	I
Stycast 2651	I	I	I	I
RTV-40	I	I	I	I
Stycast 2850	I	I	I	I

TABLE III-9
TOXICITY OF VARIOUS SOLVENTS TO B. SUBTILIS VAR. NIGER
IN TRYPTICASE SOY BROTH

+ = Growth; - = No Growth

Solvent	Spore Conc.	Time Elapsed					
		15 hr	16 hr	19 hr	21 hr	23 hr	39 hr
Benzene	10^6	+	+	+	+	+	+
Toluene	10^6	-	-	+	+	+	+
Tetra Hydrofuran	10^6	-	-	+	+	+	+
Tetraethylene Pentamine	10^6	-	-	-	-	-	-
Ethylene Diamine	10^6	-	-	-	-	-	-
Dimethyl Formamide	10^6	-	-	+	+	+	+
Pyridine	10^6	+	+	+	+	+	+

TABLE III-10
TOXICITY OF MATERIALS TO B. SUBTILIS VAR. NIGER
IN TRYPTICASE SOY BROTH

+ = Amount of Growth; - = No Growth

Material Analyzed	Spore Conc.	Time Elapsed Before Detachable Growth					
		5 hr	16 hr	19 hr	21 hr	23 hr	39 hr
<u>LIQUIDS:</u>							
Control Culture	10 ⁶	+	++	++	+++	++++	++++
Cat-A-Lac	10 ⁶	-	-	-	-	-	+
Catalyst 443-1-500	10 ⁶	0	+	+	+	+	+
Eccogel 1265A	10 ⁶	-	-	-	-	+	+
Eccogel 1265B	10 ⁶	-	-	-	-	-	-
Epon B-1	10 ⁶	-	-	-	-	-	-
Epon B-3	10 ⁶	-	-	-	-	-	-
Eccocoat IC2-A	10 ⁶	-	-	-	-	-	+
Eccocoat IC2-B	10 ⁶	-	-	-	-	-	-
Diluent IC2	10 ⁶	-	-	-	-	+	+
Catalyst 9	10 ⁶	-	-	-	-	-	-
Catalyst 11	10 ⁶	-	-	-	-	-	+
Acetone	10 ⁶	-	-	+	+	+	+
<u>SOLIDS:</u>							
Epon 901	10 ⁶	-	-	-	-	-	+
Stycast 2850	10 ⁶	-	-	-	-	-	+
Stycast 1090	10 ⁶	-	-	-	-	-	+
Stycast 2651	10 ⁶	-	-	-	-	-	+
RTV-40	10 ⁶	-	-	-	-	-	+

TABLE III-11

DETECTION OF MICROORGANISMS UNIFORMLY INOCULATED THROUGHOUT
VARIOUS SOLIDS (PULVERIZED BY DRILLING)

B = B. subtilis var. niger; S = Cl. sporogenes; U = Ulocladium; C = Contaminants

Level of Inoculum	Solids Tested				
	Piccotex 120	Stycast 2850 GT	RTV-40	Stycast 2651	Epon 901
0	0/5	0/5	0/5	0/5	2/5
10	0/5	0/5	0/5	1/5C	0/5
10 ²	5/5C	0/5	0/5	0/5	0/5
10 ⁴	0/5	0/5	5/5B	0/5	0/5
10 ⁶	0/5	0/5	0/5	1BC/5	5B/5

TABLE III-12
DETECTION OF MICROORGANISMS UNIFORMLY INOCULATED THROUGHOUT
VARIOUS SOLIDS (PULVERIZED BY DRILLING)

B = B. subtilis var. niger; S = Cl. sporogenes; U = Ulocladium; C = Contaminants

Level of Inoculum	Solids Tested				
	Eccogel 1265/A	Stycast 1090	RTV-40 + 10% RTV 921	Cat-A-Lac	Eccocoat IC2/A
0	0/5	0/5	0/5	0/5	0/5
10	0/5	0/5	0/5	0/5	0/5
10 ²	0/5	0/5	0/5	0/5	0/5
10 ⁴	0/5	0/5	0/5	0/5	0/5
10 ⁶	0/5	0/5	0/5	0/5	0/5

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TABLE III-13

DETECTION OF MICROORGANISMS UNIFORMLY INOCULATED THROUGHOUT
VARIOUS SOLIDS (ORGANISMS FREED FROM SOLID BY DISSOLVING IN
APPROPRIATE SOLVENT)

B = B. subtilis var. niger; S = Cl. sporogenes; U = Ulocladium; C = Contaminants
- because of no suitable solvent assays not performed

Level of Inoculum	Solids Tested				
	Piccotex 120	Stycast 2850 GT	RTV-40	Stycast 2651	Epon 901
0	-	-	-	-	0/5
10^1	-	-	-	-	0/5
10^2	-	-	-	-	0/5
10^4	-	-	-	-	0/5
10^6	-	-	-	-	0/5

TABLE III-14
DETECTION OF MICROORGANISMS UNIFORMLY INOCULATED THROUGHOUT
VARIOUS SOLIDS (ORGANISMS FREED FROM SOLID BY DISSOLVING IN
APPROPRIATE SOLVENT)

B = B. subtilis var. niger; S = Cl. sporogenes; U = Ulocladium; C = Contaminants

- because of no suitable solvent assays not performed

Level of Inoculum	Solids Tested				
	Eccogel 1265/A	Stycast 1090	RTV 40 + 10% RTV 921	Cat-A-Lac	Eccocoat IC2/A
0	0/5	-	-	-	0/5
10	0/5	-	-	-	0/5
10 ²	0/5	-	-	-	0/5
10 ⁴	0/5	-	-	-	1 BC/5
10 ⁶	0/5	-	-	-	1 BC/5

TABLE III-15
DETECTION OF MICROORGANISMS AT AN INTERFACE WITH THE ALUMINUM CAP
AND THE SOLID (SITE OF INOCULUM WAS EXPOSED TO CULTURE MEDIA)

B = B. subtilis var. niger; S = Cl. sporogenes; U = Ulocladium; C = Contaminant

Level of Inoculum	Solid Tested				
	Piccotex 120	Stycast 2850 GT	RTV 40	Stycast 2651	Epon 901
0	0/5	0/5	0/5	0/5	0/5
10	0/5	0/5	0/5	0/5	0/5
10 ²	0/5	0/5	0/5	0/5	1B/5
10 ⁴	0/5	0/5	0/5	0/5	0/5
10 ⁶	0/5	0/5	0/5	0/5	0/5

TABLE III-16
DETECTION OF MICROORGANISMS AT AN INTERFACE WITH THE ALUMINUM CAP
AND THE SOLID (SITE OF INOCULUM WAS EXPOSED TO CULTURE MEDIA)

B = B. subtilis var. niger; S = Cl. sporogenes; U = Ulocladium; C = Contaminant

Level of Inoculum	Solids Tested				
	Eccogel 1265/A	Stycast 1090	RTV 40 + 10% RTV 921	Cat-A-Lac	Eccocoat IC2/A
0	0/5	0/5	0/5	0/5	0/5
10	0/5	0/5	0/5	0/5	0/5
10 ²	0/5	1B/5	0/5	0/5	0/5
10 ⁴	0/5	0/5	0/5	0/5	0/5
10 ⁶	0/5	0/5	0/5	0/5	0/5

TABLE III-17
DETECTION OF MICROORGANISMS AT AN INTERFACE BETWEEN AN IMBEDDED
ALUMINUM DISC AND THE SURROUNDING SOLID MATERIAL (PULVERIZED BY
DRILLING)

B = B. subtilis var. niger; S = Cl. sporogenes; U = Ulocladium; C = Contaminant

Level of Inoculum	Solids Tested				
	Piccotex 120	Stycast 2850 GT	RTV 40	Stycast 2651	Epon 901
0	0/5	0/5	0/5	5C/5	0/5
10	0/5	0/5	0/5	0/5	0/5
10 ²	0/5	0/5	0/5	0/5	0/5
10 ⁴	0/5	1B/5	0/5	0/5	5B/5
10 ⁶	0/5	0/5	0/5	0/5	3B/C/5

TABLE III-18

DETECTION OF MICROORGANISMS AT AN INTERFACE BETWEEN AN IMBEDDED ALUMINUM DISC AND THE SURROUNDING SOLID MATERIAL (PULVERIZED BY DRILLING)

B = B. subtilis var. niger; S = Cl. sporogenes; U = Ulocladium; C = Contaminant

Level of Inoculum	Solids Tested				
	Eccogel 1265/A	Stycast 1090	RTV 40 + 10% RTV 921	Cat-A-Lac	Eccocoat IC2/A
0	0/5	0/5	0/5	0/5	0/5
10 ¹	0/5	0/5	0/5	0/5	0/5
10 ²	0/5	1C/5	0/5	1C/5	1C/5
10 ⁴	0/5	3B/5	0/5	0/5	0/5
10 ⁶	0/5	5B/5	0/5	0/5	0/5

D. DISCUSSION

The recoveries of microorganisms from solids studied were all quite low. In part these results were due to the growth inhibitory effects of the solvents used in the solubilization of the solids. Freezing and thawing microorganisms cause damage to cell walls. The extent of this effect was not independently determined. The pulverization methods also had an effect upon the recovery of microorganisms. The Dremel drill appears to be the most useful of the methods studied. The shearing and cutting forces of this method of pulverization seem to be less injurious than the crushing effects of the ball mill or the crushing, shearing effects of the mortar and pestle. Unfortunately, there is no indication as to whether the relatively low recoveries were also due in part to the production of local heat. It would appear that heat, pressure and cutting forces of the Dremel drill may cause this method to be far from optimal as a method of pulverization even though it provided the best recoveries of the various methods tested.

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PHASE IV. Determination of the Reliability of Recovery of
Microorganisms from Solid Materials by Means
of Drilling Technique

The drilling and culturing techniques found most suitable in Task III were tested for reliability in Task IV. The reliability of recovery of inoculated spores of B. subtilis var. niger was studied utilizing seventy-five cultural determinations at 37°C and an equal number at 25°C. The recoveries were highest when the inoculum was distributed uniformly throughout the solid and lowest when the inoculum was distributed at the interface of either the aluminum disc or lid. The recoveries utilizing drilling and cultural techniques have been disappointingly low and are likely due to a number of factors which are discussed in the body of this report and in the Addendum.

IV. PHASE IV. DETERMINATION OF THE RELIABILITY OF RECOVERY OF MICROORGANISMS FROM SOLID MATERIALS BY MEANS OF DRILLING TECHNIQUE

A. INTRODUCTION

The objective of Phase IV is to establish the reliability of recovery of microorganisms inoculated into selected solid materials. Seventy-five determinations were attempted with each material in order to provide a statistically reliable number of trials.

The reliability of the recovery methods established in Phase III using the drilling technique and the inoculum sites are previously discussed in Phase III and illustrated in Figure II-1 was established. In place of using a mixed inoculum in this experiment, spores of B. subtilis were the only microorganisms studied.

B. METHODS

The organisms were grown and assayed as described in Phase III. All assays were performed within one day of employing the organisms as inocula. The inocula were incorporated with the materials tested in the same location and manner as previously discussed in Phase III.

In order to avoid systematic errors relating to the sequence in which analyses were performed, all three forms of inoculum distribution were used in daily analyses. Every attempt was made to maintain the same technique and in particular to control the rate and extent of pulverization of the samples. It was noted, however, that local heating took place during the course of drilling. Although the exact amount of heating that took place was not determined, its presence was nevertheless detected.

C. RESULTS

The recovery of organisms introduced as inocula into the various samples are reported in Tables IV-1, IV-2, IV-3. The largest number of microorganisms were recovered from solids in which B. subtilis spores were uniformly distributed throughout the solid. The smallest number of

TABLE IV-1

DETERMINATION OF RELIABILITY OF ASSAY SENSITIVITY

SAMPLES OF EACH SOLID TESTED TO DETERMINE REPRODUCIBILITY OF
RECOVERY OF 6×10^8 SPORES B. subtilis/Gm
UNIFORMLY DISTRIBUTED THROUGHOUT SOLID

<u>Material</u>	Tube Assay		Plate Assay	
	<u>37°C</u>	<u>25°C</u>	<u>37°C</u>	<u>25°C</u>
Stycast 2850 GT	0/75	0/75	0/75	0/75
RTV-40	1/75	0/75	3/75	0/75
Epon 901/B3	4/75	5/75	2/75	4/75
Stycast 1090	0/75	0/75	0/75	0/75
Eccocoat IC2	30/75	9/75	12/75	9/75

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TABLE IV-2

DETERMINATION OF RELIABILITY OF ASSAY SENSITIVITY

SAMPLES OF EACH SOLID TESTED TO DETERMINE REPRODUCIBILITY OF
RECOVERY OF 6×10^8 SPORES B. subtilis/Gm
INOCULUM AT INTERFACE OF LID AND SOLID

<u>Material</u>	Tube Assay		Plate Assay	
	<u>37°C</u>	<u>25°C</u>	<u>37°C</u>	<u>25°C</u>
Stycast 2850 GT	0/75	0/75	0/75	0/75
RTV-40	2/75	0/75	1/75	0/75
Epon 901/B3	5/75	0/75	0/75	1/75
Stycast 1090	0/75	0/75	0/75	3/75
Eccocoat IC2	6/75	3/75	5/75	0/75

TABLE IV-3

DETERMINATION OF RELIABILITY OF ASSAY SENSITIVITY

SAMPLES OF EACH SOLID TESTED TO DETERMINE REPRODUCIBILITY OF
 RECOVERY OF 6×10^8 SPORES B. subtilis /Gm
 INOCULUM LOCATED AT INTERFACE OF ALUMINUM DISC AND SOLID

<u>Material</u>	Tube Assay		Plate Assay	
	<u>37°C</u>	<u>25°C</u>	<u>37°C</u>	<u>25°C</u>
Stycast 2850 GT	2/75	1/75	0/75	0/75
RTV-40	0/75	0/75	1/75	0/75
Epon 901/B3	0/75	1/75	2/75	1/75
Stycast 1090	0/75	1/75	0/75	0/75
Eccocoat IC2	3/75	0/75	2/75	1/75

microorganisms recovered was when the inoculum was located at the interface of the aluminum disc and the solid (Table IV-3). The growth of microorganisms was more rapid at 37°C than at 25°C. In most instances the tube assay appeared to be superior in recovery to the plate assay.

D. DISCUSSION

It is apparent that the recoveries of sizable inocula by the use of drilling techniques were disappointingly low. The possible reasons for such low recoveries may be manifold. In the course of pulverizing the samples the organisms residing at the various locations in the solids may have been ineffectively exposed. This appears to be likely in view of the fact that the particle distribution of plastic materials similar in nature to those studied indicated that the ideal particle size was neither uniform nor sufficiently small to free the encased organisms. Furthermore, in attempting to reconcile these results with those found later in our laboratory, it was discovered that the particle size distribution obtained during pulverization of plastic materials varied as a function of the pressure applied while drilling, the sharpness of the drill, and the operator.

The temperature rise during the course of drilling varied according to the area being drilled. The distribution of the inocula included the following: uniformly distributed throughout, applied to the interface between the solid and the aluminum lid, and at the interface of an aluminum disc and the solid material.

Recent experiments conducted by the authors to establish why recoveries of large populations of microorganisms were so disappointingly low, indicate that in addition to pulverization parameters, the presence of toxic materials within the plastic and in some instances the toxicity of the solvents play extremely significant roles. Some of our more recent work on solids in which attempts have been made to minimize these effects are discussed in the Addendum which follows.

E. CONCLUSIONS

Regardless of the site of inoculum, whether uniformly mixed throughout the solid, applied at an interface between the solid and the lid, or applied at the interface of an aluminum disc and the solid, the recovery of microorganisms used as inocula was uniformly unsatisfactory. There appeared to be differences in the recoveries of microorganisms from the various solids. In addition, it appeared that best recoveries were obtained when the microorganisms were distributed uniformly throughout the solid. In the instances in which the microorganisms were deposited at the interface between the lid and the solid, and between the face of the aluminum disc and the solid, the recoveries were considerably lower.

ADDENDUM

The interim between the completion of the work reported in Phases I through IV of this program and the writing of this addendum, improvements on the dilution methods for microorganisms in solid materials have been made. These have involved extension in the range of sensitivity of detecting viable microorganisms to 10^2 microorganisms per cm^3 of solid material and in some instances to as low as 10 microorganism/ cm^3 .

The improvements in detection first took place in the study of microorganisms in solid propellants. While conducting these investigations it became apparent that certain modifications must be made in the recovery techniques in order to increase the sensitivity of detection of microorganisms. We found certain inhibitory substances in the propellant that prevented the maximum growth of our inoculum. These inhibitors had to be leached out to increase recovery efficiency. It was possible, for example, to not detect as many as 10^4 microorganisms per cm^3 in solid propellant if the leaching step was omitted. When the leaching step and certain additional steps were included, it was possible to detect as few as 10 microorganisms per cm^3 .

Improvements were also made in the pulverization of the solid. Through the use of saws possessing certain specific characteristics, it was possible to pulverize solid propellant more effectively than any other previously used method. A thermistor was attached to the back one inch of blade and a slow rate of sawing was used. The rate of sawing was so regulated that an increase in temperature of not more than 1°C resulted. Because the temperature at the saw tooth-propellant interface may be several degrees higher than that indicated by the thermistor, the rate of sawing was adjusted to minimize possible thermal inactivation of microorganisms. The procedures are summarized in Figure A-1.

Microscopically innumerable fine cracks and checks were noted on the sawed particles which were noted to have continuity with the interstices of even the largest particles. The larger particles appeared to result from

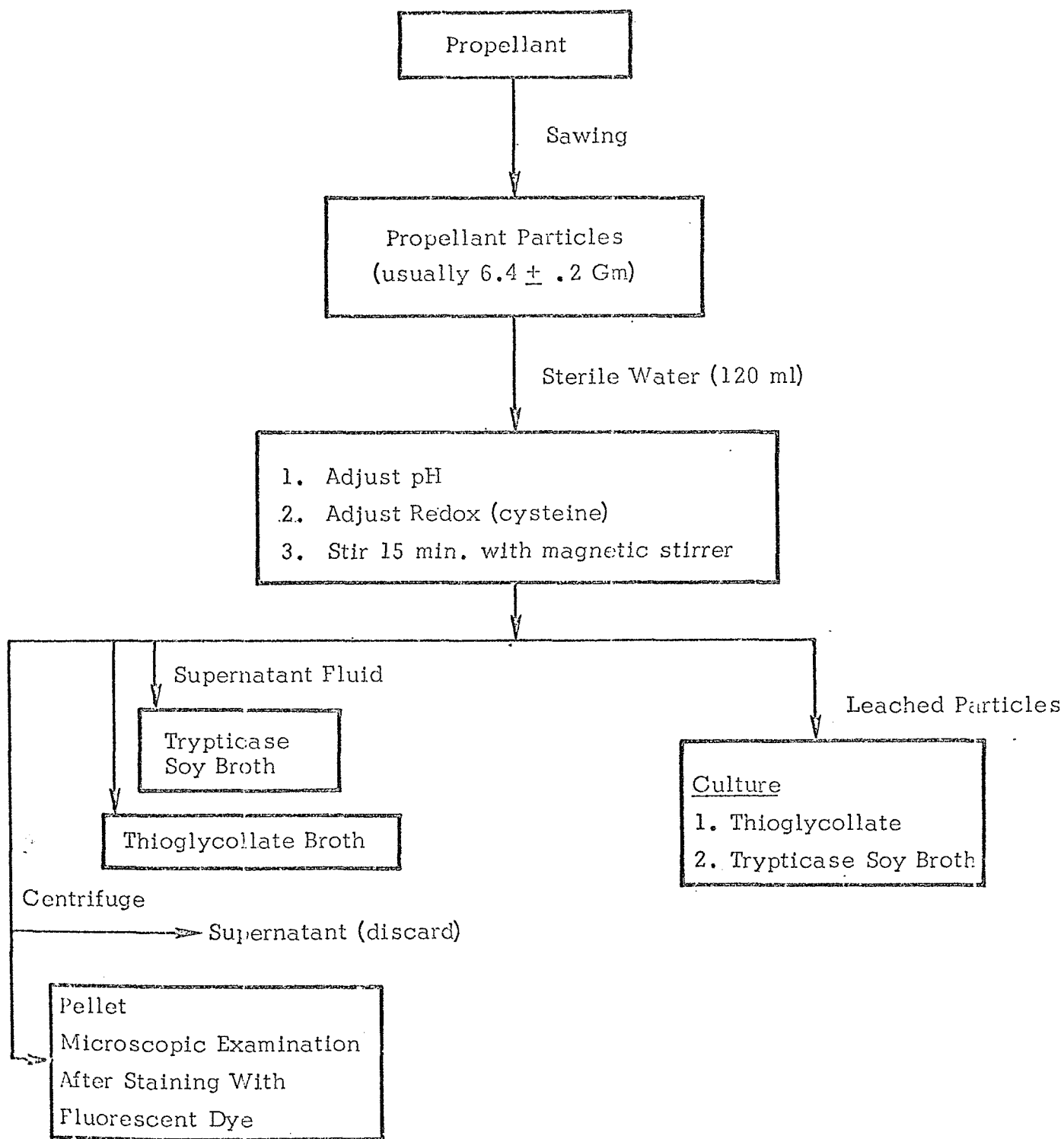


Figure A-1. Propellant Culturing Procedure

the fusion of smaller particles. This fusion effect was likely due to the sticky adherent surfaces of the solid propellant material. This fusion effect could not be reversed by treatment with the Waring blender, sonication, or by the treatment with wetting agents. Although Tween-80* was included in the culture media and did provide superior recoveries, it does not appear that such improved recoveries were the result of inhibition of particle agglomeration.

Leaching of Propellant

The propellant particles were extracted with sterile distilled water and stirred with a teflon coated magnetic stirring bar under sterile conditions. The optimum ratio of leaching fluid to solid propellant was established. The redox potential of the leached propellant and the supernatant fluid was measured and this potential was adjusted to that considered optimum for each microorganism tested. The supernatant fluid at the end of the leaching period was removed, the pH adjusted to 7.2 - 7.4 using sterile 0.1 N acid or base (except in the case of Ulocladium or other fungi the pH was adjusted to 4.0 - 4.5). Tween-80 was incorporated in the growth medium (Figure A-2).

A Reappraisal of Assay Techniques in Solid Materials

The application of steps previously noted in this addendum have increased the sensitivity of the detection of inoculated organisms from a level of 10^5 to 10^1 per cm^3 . Recovery of microorganisms from inoculated solid propellant utilizing microtome sectioning technique was uniformly poor as noted previously in Phase III discussion.

Because of the large order magnitude of improvement in the recovery of small members of microorganisms from propellant, our studies were extended to solids from which previously large inocula of organisms ($10^8/\text{gram}$)

*polyoxyethylene-sorbitan-monooleate (Atlas Powder Co.)

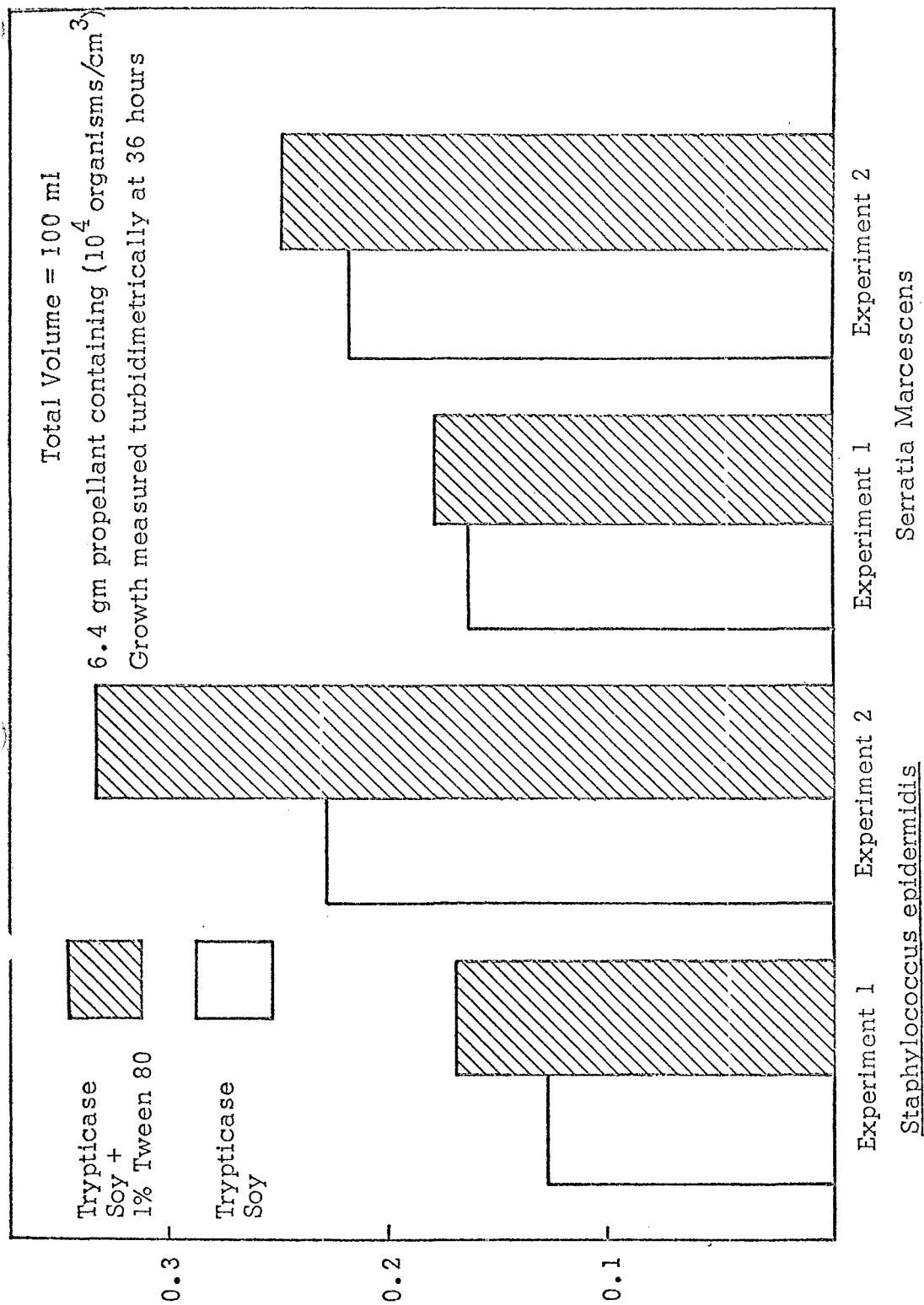


Figure A-2. Effect of Tween 80 on Growth of Selected Bacteria in Propellant as Determined Turbidimetrically

gave in many instances negative recoveries of microorganisms. By applying these improved methods, it was possible to detect as few as 100 microorganisms per cm^3 from two types of epoxy resins. It therefore is apparent that the culture techniques originally developed for solid propellants are applicable in the detection of microorganisms in solid plastic materials (Stycast 2850 GT, Eccocoat IC2) from which recoveries were previously unsatisfactory.

Recommendations

In extending the assay procedures which were devised and optimized for propellant recovery studies, it would be desirable to study in greater detail the pulverization of the various classes of solid materials. If the pulverization was so drastic as to cause the rupturing of the cell wall in the microorganism, it would be likely that the organism could be detected by cultural techniques. The process of freeing the microorganisms from encasement in rigid solids may tend to cause irreversible cellular damage. Although the particle size achieved by the pulverization may approach that of the microorganism, this process may be less desirable than one which produces larger particles.

In the latter case, communicating microcracks may exist which would provide both for diffusion of nutrients to and growth of the encased microorganisms from internal sites.

Because of the occurrence of toxic compounds in certain types of solid materials, it will be important to establish optimal methods for the leaching of these substances free from the pulverized solid. A large variety of plastic polymers are polymerized by catalysts which may inhibit the growth of microorganisms. These catalysts vary according to the type of plastic and each represents a separate problem. Unreacted monomers when present may also inhibit the growth of microorganisms. It is obvious that in addition to optimizing the pulverization and leaching procedures that it will be necessary to devise methods for neutralizing or inactivating both the soluble leachable inhibitors and the inhibitors present within the solids themselves.

Further investigation of culture media development will be required to optimize the isolation of microorganisms from solids. Since the type of biochemical injury or event which results in the devitalization of the microorganism may not be definable, it may be necessary to add a variety of substances which tend to counteract these events. In order to reactivate such inhibited organisms it may be necessary to add growth factors and metabolic constituents to the medium which may not normally be required by the organism under other conditions. The addition of an excess of the known required vitamins, amino acids, sugars and growth factors at levels which do not interfere with growth or metabolism of the organism is highly desirable. Adjustment of trace metal content, ionic strength, pH and redox potential may also be desirable.

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MICROORGANISMS IN SOLID MATERIALS

TASK I:

RESISTANCE OF ALPHA ORGANISMS TO DRYING AND TO
STERILIZATION BY ETHYLENE OXIDE

(Mod. 1)

VOLUME III

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SUMMARY

Alpha has been identified as a variant of species Staphylococcus epidermidis. Organisms of this species are the most prevalent of micro-organisms on all surfaces of human skin. They are also prevalent in the human mouth, nose, and respiratory tract. They are of low pathogenicity. The human being is not, however, the only source of Staphylococcus contamination.

Characteristically, organisms of the Staphylococcus species survive exposure to a wide variety of chemical agents and tolerate high concentrations of salts in liquid media. They are not resistant to heat as are bacterial spores. They appear to be sensitive to changes in their water content and the environment in which these changes are brought about. From some natural sources, populations of Staphylococcus organisms can be dried with preservation of most of the cells in a viable state. Drying from suspension in pure solvents destroys detectable viability in even large populations. Once the cells have been properly dried, however, they appear to remain viable for relatively long periods of time and to be resistant to the action of a variety of chemical agents.

Because of their agglomerating nature, quantitative assay of the number of viable cells in a population of Staphylococcus organisms is difficult.

I. INTRODUCTION

In assaying the internal microbial population of a specimen of solid propellant (Opfell, 1962 a and b) which had been prepared containing aluminum particles, liquid ethylene oxide, and an inoculum of spores of B. subtilis var. niger, the microorganism alpha was isolated. Because it survived in an environment which destroyed the detectable viability of the B. subtilis var. niger spores, alpha is of interest in demonstrating the effectiveness of processes for the preparation of sterile solid propellant.

Alpha and alpha-like species have been isolated subsequently from specimens of dust (Opfell, 1963a) from the JPL spacecraft assembly facility and from subsequent specimens of solid propellant (Opfell, 1964b).

The following paragraphs will describe and interpret the results of experiments to determine the ethylene-oxide resistance of populations of alpha in a variety of environments and after several types of pre-treatment. The evidence on which the classification Staphylococcus epidermidis has been assigned to this species will be presented and its significance in the engineering design of sterilization steps will be discussed.

The study described here represents a continuation of a recent study (Opfell, 1964a) to evaluate and to establish methods for assaying the viability of microbial populations dispersed in the interior of solid materials. A parallel study (Opfell, 1964b) and a previous study (Opfell, 1962a and b) applied these techniques to solid propellants. In both these studies populations of the species alpha or of species similar to it were found in the propellant specimens.

II. THE IDENTITY OF SPECIES ALPHA

On the basis of tests reported in Table I and comparison with the properties of ATCC strain 155 of Staphylococcus epidermidis, alpha has been identified to be a strain of Staphylococcus epidermidis. Though the distinction from species of Gaffkya is not as clear as one might like, the reasons for not calling alpha a species of Gaffkya are presented in Table II. As the discussion which follows will show, this distinction may not be really possible. Some bacteriologists (Stanier, 1963) call the staphylococci the pathogenic-members of the genus Micrococci and in particular use the term Micrococcus pyogenes, var. aureus for Staphylococcus aureus and Micrococcus pyogenes, var. albus for Staphylococcus albus.

Jones (1963) has studied fifteen strains of Staphylococcus epidermidis and found that some, like members of the genus Gaffkya, failed to reduce nitrates. One of the strains had a colored pigment. Jones lists the important characteristics of Staphylococcus epidermidis to be:

- 1) ability to grow anaerobically in a defined medium with glucose or pyruvate as energy source
- 2) inability to produce coagulase
- 3) ability to ferment serine
- 4) requirement for biotin and uracil under anaerobic conditions in a semisynthetic medium
- 5) inability to reduce nitrate beyond nitrite

Deibel (1960) proposed a set of characteristic properties for the genus Pediococcus. Adoption of this recommendation would eliminate the genus Gaffkya (U. S. Naval Medical School). The U. S. Naval Medical School (1963) points out the difficulty in distinguishing Gaffkya isolates from Staphylococcus epidermidis (coagulase negative). Gaffkya is distinguished by a high frequency of tetrads among cells isolated from

body fluids and by failure to reduce nitrate to nitrite. Many species of Micrococcus fail to reduce nitrate to nitrite (Skerman, 1959) and many including Staphylococcus epidermidis (also alpha) forms tetrads under several conditions of culture. Gaffkya can be easily distinguished from Staphylococcus aureus, however, by the nitrate and coagulase tests. Burrows (1954) assigns the name Micrococcus tetragena to Gaffkya tetragena, and shows a picture of a smear from pure culture. The picture would serve equally well as a description of alpha.

Skerman (1959) points out that considerable difficulty may be encountered in differentiating between the genus Micrococcus and the genera Staphylococcus and Pediococcus. He states that the genus Staphylococcus has been allocated only two species, Staphylococcus aureus and Staphylococcus epidermidis, omitting any mention of Staphylococcus albus. He suggests that the characteristics which differentiate the genus Pediococcus are based on byproducts produced in certain media and that comparable studies of the byproducts produced by the genus Staphylococcus and the genus Micrococcus have not been made.

Cowan (1964) suggests that catalase-positive Gram-positive cocci that ferment glucose under anaerobic conditions can be regarded as Staphylococci and that those which fail to do so are not Staphylococci -- they may be other species of Micrococci. He suggests also that Staphylococci be divided into two species: Staphylococcus aureus (coagulase-positive) and Staphylococcus epidermidis (coagulase-negative).

Bryan (1953) describes another species, Staphylococcus aerogenes, which is a strict anaerobe and might not apparently be a member of the genus in the terms defined by Cowan. Bryan says of Staphylococcus epidermidis that it is but Micrococcus epidermidis by another name and that it may be a less virulent variety of Staphylococcus albus. Frobisher (1962), on the other hand, equates the names Staphylococcus albus and Staphylococcus epidermidis.

III. THE HABITAT OF SPECIES ALPHA

The foregoing discussion shows the futility of inferring the range of habitat of alpha from the published literature on Staphylococcus epidermidis alone. Also, the confusion in the taxonomy of Staphylococcus epidermidis with that of similar species suggests that information on the habitats of other genera in the family Micrococcaceae or the tribe Streptococceae would be useful in estimating the significance of alpha in the environment surrounding the manufacture of sterile solid propellant. Among these genera are Micrococcus, Staphylococcus, Gaffkya, Sarcina, and perhaps even Pediococcus. Streptococceae organisms are found regularly in the mouth and intestinal tract of humans. In the mouth and respiratory tract Streptococceae cells are more prevalent (U. S. Naval Medical School, 1963) than are cells of the Micrococcaceae genera. On the exterior body surfaces, however, the Staphylococci are by far the most prevalent cells. Roseberg (1963) and Wilson (1955) point out that coagulase-negative staphylococci (Staphylococcus epidermidis) are prevalent on all areas of human skin. Roseberg also states that human skin normally carries a population of Clostridia and anaerobic cocci. Because growth of both Streptococceae populations and Clostridia populations is poor or absent on many of the media employed in the present tests, they might have been undetected in the propellant ingredients which were analyzed.

Gregory (1961) discusses reports of finding viable cells of Staphylococcus aureus and of Staphylococcus albus in the air over the open ocean. He suggests, however, that these populations came from land sources. The ability of cells of Staphylococcus species to survive in environments outside of living animals depends upon a variety of factors, many of which have not yet been explored.

An alpha population stored in purified water decays in a manner of multicellular organisms (Umbreit, 1962) which in a sense they are. A relatively small portion of the cells in a population of Staphylococcus cells occur singly. Most are in pairs, tetrads, or small clusters which do not break up easily.

IV. CLONAL VARIATION

Burrows (1954) points out that there is good reason to believe that bacterial populations are heterogenous with respect to the ability of the individual cells to withstand the effects of lethal environments. The implications of this hypothesis has been developed as a theory of clonal variation and selection. Heterogeneity is accounted for by the assumption that binary fission of a bacterial cell does not always produce daughter cells of precisely equal resistance to each of the lethal factors in the environment. The occurrence of two daughter cells of equal resistance is the most probable result of fission, of two daughter cells of slightly different resistance, slightly less probable, and of two daughter cells with much greater resistance, much less probable. Experience with the development of antibiotic resistance in strains of Staphylococcus aureus constitutes a large portion of the evidence in support of the theory of clonal variation.

When cultural conditions are changed, increasing numbers of the less resistant daughter cells may fail to reproduce with the result that over several generations, the average resistance of the cells in the culture has increased. With a return to the original cultural conditions, the resistance of the cells in the population will return to the original distribution of resistances after the passage of several generations. The populations of Staphylococcus epidermidis strains appear to be susceptible to this type of clonal variation. Staphylococcus epidermidis sp. x arose from the storage of a water suspension of alpha in the refrigerator for a year and a half.

V. ASSAY OF STAPHYLOCOCCUS EPIDERMIDIS

The variations in population growth rate among strains and even replicates of the same culture of a strain of Staphylococcus epidermidis is illustrated in Table III. The reason for the variations lies partially in the multicellular (Umbreit, 1962), form in which the viable particles of Staphylococcus epidermidis occur. Serial dilution breaks these particles apart and thereby extends the infectivity to greater dilutions through increasing number of independent viable particles. The greater the dilution, the greater the possibility for error in estimating the number of viable particles.

The grouping of the cells of Staphylococcus epidermidis has an effect on the ability of populations of them to withstand drying. The viable cells find themselves attached to colloidal particles (other cells, both living and dead for example). Umbreit (1962) points out that when bacteria are absorbed onto the surface of finely divided particles (colloid), their metabolic activity often slows down and they seem to survive much longer. After being so absorbed they are often resistant to drying. Protein materials also have a strong preservative effect during drying of many bacteria and viruses. No attempt was made to measure the effect of drying inoculums on the colloidal size particles of ammonium perchlorate (the principal ingredient in solid propellant) on the survival of cells in these inoculums. The water in the inoculum would dissolve ammonium perchlorate and on further drying the cells would be occluded in the ammonium perchlorate. The obvious test would not necessarily separate the colloidal effects from other effects of the ammonium perchlorate particles.

VI. RESISTANCE OF ALPHA POPULATIONS TO THE EFFECTS OF SEVERAL ENVIRONMENTAL FACTORS

On the surface of Trypticase soy agar at 37°C, alpha populations were found to remain viable for periods in excess of nine days. During this time the agar dried enough to become crisp. When alpha cells were collected, washed, and suspended in previously sterile purified water, the assay of viable cells did not change on storage of the suspension for periods up to a week. On eight-days' storage, however, the numbers of particles in the viable population appeared to drop by four to five orders of magnitude. After this drop in viability, the number of viable cells continued to fall but more slowly with some cells in the suspension remaining viable for very long periods of time. The viable population of Staphylococcus epidermidis ATCC 155 in water behaved similarly. No attempt was made to measure the ethylene-oxide or drying resistance of the survivors.

Table IV shows the comparative effects of suspension of viable cells of a species of Staphylococcus epidermidis and of spores of B. subtilis var. niger in several pure solvents. The toxicity of acetone for alpha populations was confirmed by the experiments reported in Tables VI, X, XI, and XII. Each of these three solvents are strong desiccants and likely withdraw water from the Staphylococcus cells in a manner other than optimum for preserving viability. That cells of Staphylococcus species are sensitive to their environment during desiccation is shown also by the experiment reported in Table IX and the studies reported by McDade (1963) and by Skaliy (1964). The presence of substances from agar or broth helped preserve viability in populations discussed in Table IX. McDade found that Staphylococcus aureus populations had variable resistance to drying under the synthetic conditions he used. His populations of about 1000 cells in broth were dried on sutures in an environment of air at several levels of relative humidity. Even at 50% RH, the condition most destructive of viability, his populations remained viable up to four days. Skaliy, on the other hand, found that one day was sufficient

for a Staphylococcus aureus population in a hospital room to be undetectable after the departure of a patient who had been confined there with a Staphylococcus aureus infection. Sykes (1958) reports that Staphylococcus aureus is the most resistant to drying of members of the Staphylococcus genus.

Using a cylinder-plate assay technique Snell (1954) measured the degree to which several organic solvents inhibit the growth of populations of several species of bacteria on an agar surface. Among the organic solvents tested were the three listed in Table III. Snell found one of the three to inhibit Micrococcus pyogenes, var albus (possibly, though not likely, the same strain as alpha) though both absolute ethanol and acetone inhibited Sarcina lutea, another species of the family Micrococcaceae. The conditions of exposure in Snell's study were substantially different from those used in the experiments corresponding to Table III. The desiccating effect of the solvent in Snell's cylinder-plate assay should have played a negligible role whereas it was possibly the dominant effect in the Table III experiment.

VII. TOXICITY OF PROPELLANT INGREDIENTS

The ability of a broth to support growth of a population of micro-organisms in the presence of a toxic ingredient varies from batch to batch. In the tests reported in Table V, for example, an inoculum of 10^4 spores of *B. subtilis* var *niger* was able to grow in the presence of 0.5% v/v ethylene oxide. The three propellant ingredients, TDI, PPG, and AMP failed to suppress growth of any of the species tested even when the broth was saturated with them.

The toxicity of the propellant ingredients for alpha depends on the state of the alpha organisms at the time they are introduced into contact with the ingredient. In the tests reported in Table VI, the alpha organisms in 0.01 ml of purified water were placed in the bottom of previously sterile test tubes. The inoculum was permitted to dry, though not completely. (Some inoculums may have dried completely while others were seen to contain water at the time the propellant ingredients were placed on top of them). The exposure to ethylene oxide was made by placing the open tubes containing the inoculum and the propellant ingredient in a 4-ml polyethylene bag containing an atmosphere of air saturated with ethylene oxide vapor and water in excess of that equivalent to 50% RH. The tubes were exposed to this environment for 2 hours. The gas was flushed around the inside of the bag and over the tops of the tubes several times during the exposure period.

After removal from the bag the contents of each tube, including those not exposed to ethylene oxide, were stirred with a separate, sterile, glass stirring rod. The tubes were then stored at room temperature for the remainder of a 24-hour exposure period. After the end of this period, 5 ml of sterile fluid thioglycollate medium was put into each tube. Into each of the bacteriostasis control tubes were put 0.1 ml of purified water suspension of either alpha cells or spores of *B. subtilis* var *niger* containing approximately 100 viable particles each.

The tubes of broth were then incubated at 37°C for 7 days. A loopful of the broth was then streaked on the surface of Trypticase soy agar in a Petri dish. These streak plates were then incubated under the same conditions that the broths had been.

Generally, the propellant ingredients made the broths appear turbid so that microbial populations in the broth could not be detected on the basis of the appearance of the broth alone. The bacteriostasis tubes containing B. subtilis var. niger, however, contained an orange pellicle when the broth was not bacteriostatic.

In only one case, did the alpha population survive the sequence of conditions met in the test. This result is in contrast with the results shown in Table V for three of the ingredients. Moreover, the propellant ingredients appear to have made the broths bacteriostatic for inoculums of alpha containing but 100 viable particles in purified water. The inoculums used in the experiments corresponding to Table V included 10^4 viable particles.

The failure of the B. subtilis var. niger inoculums to grow in the broths containing Alroperse (a reaction product of an equal mixture of stearic acid and diethynolamine) or to grow vigorously in those containing FeAA, unless these materials had been exposed to ethylene oxide is surprising and should be confirmed by further tests.

In view of the sensitivity of alpha to the conditions under which it is dried, the results shown in Table VI may not be surprising. Moreover, populations of 100 viable particles of Staphylococcus organisms seem to be more sensitive to toxic effects in broth than are populations greater than 10^4 . This possibly is indicated in Table VIII.

VIII. NATIVE CONTAMINATION IN THE PROPELLANT INGREDIENTS

The broths at the "O" inoculum level would have disclosed native contamination in the propellant ingredients had there been a significant amount. The bacteriostasis controls show that these broths could have detected populations of 100 spores of organisms like B. subtilis var. niger but would fail to detect similar numbers of viable particles of alpha. This result does not mean that spores are necessarily more easily detected in the smaller numbers. Tables VII and VIII show that in broths containing ethylene oxide, larger populations of B. subtilis var. niger spores than of viable particles of Staphylococci were required to produce observable changes in the detection medium.

The low order of the contamination in several of the propellant ingredients was confirmed in the test made on duplicate samples of a Pfaudler-pot fuel mix (the results and the descriptions of the tests were reported by Opfell, 1964 b). This fuel mix contained Alrosperser, PPG and TMP.

The tests used were not sufficiently sensitive to support a conclusion that the propellant ingredients were sterile. The technique used to test them was not sterile, though care was taken to avoid accidental contamination.

IX. RESISTANCE OF SEVERAL SPECIES OF BACTERIA TO ETHYLENE OXIDE

The inoculum required to produce population growth in broth containing ethylene oxide depends upon the type of broth, the concentration of ethylene oxide and the species and strain of the microorganisms. Tables VII and VIII compare the relative resistances of several species of microorganisms in both Trypticase soy broth and fluid thioglycollate medium and in the presence of a range of concentrations of ethylene oxide. In the 40 ml tubes closed with Morton closures, in which the broths were incubated, the concentration of ethylene oxide in the broth was confined to drop by no more than 50 percent on incubation at 37°C for 24 hours.

Ethylene oxide in the amount of 0.3% has been reported (Toplin, 1961) to sterilize fermentation broths. Tables VII and VIII show the levels of contamination by each of several species which might cause sterilization failure. Interestingly, the Staphylococcus strains are the most difficult (Sykes, 1958) to destroy under the conditions tested.

X. EFFECTS OF DRYING AND EXPOSURE TO ETHYLENE OXIDE ON THE VIABILITY OF STAPHYLOCOCCUS EPIDERMIDIS POPULATIONS

Populations of alpha cells and of the ATCC 155 strain of Staphylococcus epidermidis cells were compared with respect to their ability to withstand the effects of drying. The previously observed sensitivity of alpha populations to drying (Opfell, 1963 b) from suspension in purified water was used as a confirming test for alpha. The results reported in Table IX appear to support the conclusion that alpha is less resistant (Sykes, 1958) to drying than is the ATCC strain under identical conditions. Unfortunately, identical conditions are difficult to provide. The Tests 1 and 2 were performed on different days. The ATCC 155 strain cells used had been stored in purified water for a longer period of time than they had been for Test 1. The tests on the two strains were performed simultaneously, however.

The results of Tests 3 and 4 show the protective effects of nutrient substances during drying (Sykes, 1958). McDade (1963) found that populations of 1000 cells of Staphylococcus aureus dried on sutures from suspension in broth remained viable for four days and longer. Skaliy found that populations of Staphylococcus aureus from an infected patient died away in one day. Both these results appear to be consistent with the results presented in Table IX.

Alpha was isolated originally from the interior of a specimen of solid propellant. Presumably the cells got into the propellant originally accompanied by some type of protective substances. The environment inside the propellant was sufficiently protective to preserve viability for a long period of time.

Using a fractional factorial design, the relative effects of the several factors, surface supporting the inoculum during drying, the relative humidity and the oxygen content of the ambient gas during drying, the suspension vehicle, and exposure to ethylene oxide on the viability of

population of alpha were determined. Corresponding tests were performed on populations of spores of B. subtilis var. niger, which are known to survive the ethylene oxide exposure conditions used, to be unaffected by suspension in acetone, and to be insensitive to the methods of drying. The efficiency with which spores of B. subtilis var. niger can be removed from different types of surfaces is variable. Table XI presents the results of a control test on efficiency of recovery of the viable inoculum.

The details of the tests have been described in the footnotes to Table X. The concentrations of ethylene oxide and water vapor in the ambient gas during the exposure to ethylene oxide were confirmed by gas-chromatographic analysis.

The dominant factor in destroying viability of alpha populations was the drying process in the absence of protective agents. Table X shows quantitatively the frequency with which viable particles can be recovered from coupons on which viable cells had been dried. Though an attempt was made to remove the alpha inoculum from the surface of the coupon and suspend it in the broth, comparison of Table X with Tables XI and XII shows that the treatment used was not entirely effective. Because of the possibility of accidental contamination of the coupons or of the broths in which they were incubated during the withdrawal of 0.5-ml aliquots from the jar, the recovery of viable alpha organisms from the coupons which had been inoculated with distilled water suspensions and had not been exposed to ethylene oxide was more than can be explained by contamination. After the effects of drying, the type of suspension vehicle is shown to be important but the type of solid material and the nature of the ambient gas did not produce discernable effects. The inoculum dried substantially more slowly on teflon than it did on the other materials, but this effect did affect the results.

TABLE I
CHARACTERISTICS OF STAPHYLOCOCCUS EPIDERMIDIS

Characteristics	"Bergey's Manual" Strain	<u>Alpha</u>
CELLS		
Shape	0.5-0.6 micron spheres	0.5 micron spheres
Gram-staining characteristics	Gram-positive	Gram-positive
Aggregation	singles, pairs, and irregular groups	singles, pairs, tetrads, and small groups
Mobility	nonmotile	nonmotile*
CULTURES		
Optimum temperature for growth	37°C	37°C
Oxidation-potential requirements	aerobic, facultative anaerobic	aerobic, facultative anaerobic
Nutritional factors required	amino acids for nitrogen, biotin, some B vitamins	appears to use ammonium from ammonium perchlorate
Salt tolerance	grows vigorously in media containing 10% sodium chloride	grows in 8% NaCl agar*
Appearance of broth containing fermentable carbohydrate	heavy uniform turbidity	medium uniform turbidity in TSB,** long thick "fingers" of turbidity in anaerobic part of FTM, gray-white sediment
Action on special media		
Litmus milk	acid	acid*
Nitrate broth	nitrate reduced to nitrite	nitrate reduced to nitrite
Glucose in broth	acid produced	acid produced*
Fructose in broth	acid produced	
Maltose in broth	acid produced	
Trehalose in broth	acid produced	
Sucrose in broth	acid produced	acid produced
Glycerol in broth	acid produced	
Mannose in broth	acid may be produced	

*Oltzky

**Trypticase soy broth and FTM indicates fluid thioglycollate medium

TABLE I (Continued)

Characteristics	"Bergey's Manual" Strain	<u>Alpha</u>
CULTURES (Continued) Galactose in broth Lactose in broth Xylose in broth Arabinose in broth Raffinose in broth Inulin in broth Sorbitol in broth Mannitol in broth	acid may be produced acid may be produced no acid produced no acid produced no acid produced no acid produced no acid produced no acid produced	acid produced* no acid produced*
COLONIES Gelatin-stab colonies Agar-surface colonies	white surface growth with slow saccate liquifaction smooth, circular, translucent white colonies	slow liquifaction smooth, circular, translucent white colonies
OTHER CHARACTERISTICS Indole test Urease test Catalase test Pathogenic Parasitic Coagulase test Hemolysis test	catalase positive no yes coagulase negative	no indole produced* urease produced* nonhemolytic

*Olitzky

TABLE I (Continued)

Characteristic	"Bergey's Manual" Strain	<u>Alpha</u>
ORIGINAL SOURCE	human skin wounds and abscesses	a specimen of solid propellant containing aluminum powder, ethylene oxide, and an inoculum of <u>B. subtilis</u> var. <u>niger</u> spores
HABITAT	skin and mucous membranes of humans and other animals	dust at JPL - possibly also people
DISTINCTIVE CHARACTERISTICS	ferments glucose but not mannitol under anaerobic conditions coagulase negative	appearance in FTM form of colonies frequency of tetrads in smears from surface colonies ethylene-oxide resistance sensitivity to drying from suspension in pure solvents

TABLE II
CHARACTERISTICS OF ALPHA COMPARED WITH THOSE OF
GAFFKYA TETRAGENA

Characteristic	<u>Gaffkya Tetragena</u> ⁺	<u>Alpha</u>
CELLS		
Shape	1 micron sphere	0.5 micron spheres
Gram-staining characteristics	Gram-positive	Gram-positive
Aggregation	singles, pairs, and tetrads (particularly in body fluids)	singles, pairs, tetrads, and small groups
Mobility	nonmotile	nonmotile*
Capsule	pseudocapsule in body fluids	
CULTURES		
Optimum temperature for growth	37°C	37°C
Oxidation-potential requirements	aerobic, facultative anaerobic	aerobic, facultative anaerobic
Nutritional factors required	amino acids for nitrogen biotin some B vitamins	appears to use ammonium from ammonium perchlorate
Salt tolerance		grows in 8% NaCl agar*
Appearance of broth containing fermentable carbohydrate	clear with gray sediment	medium uniform turbidity in TSB,** long thick "fingers" of turbidity in anaerobic part of FTM, gray-white sediment
Action on special media		
Litmus milk	acid	acid*
Nitrate broth	does not reduce nitrate*	nitrate reduced to nitrite
Glucose in broth	acid produced	acid produced*
Fructose in broth		
Maltose in broth		
Trehalose in broth		
Sucrose in broth		
Glycerol in broth	acid produced	acid produced
Mannose in broth		

*Ollitzky

**Trypticase soy broth and FTM indicates fluid thioglycollate medium

+ Bryan

TABLE II (Continued)

Characteristic	<u>Gaffkya Tetragena</u>	<u>Alpha</u>
CULTURES (Continued) Galactose in broth Lactose in broth Xylose in broth Arabinose in broth Raffinose in broth Inulin in broth Sorbitol in broth Mannitol in broth	acid produced	acid produced no acid produced
COLONIES Gelatin-stab colonies Agar-surface colonies	white surface growth but with no liquification smooth, circular, translucent white, glistening colonies with entire edge	smooth, circular, translucent white colonies
OTHER CHARACTERISTICS Indole test Urease test Catalase test Pathogenic Parasitic Coagulase test Homolysis test Gas production	no coagulase negative none ⁺	no indole produced urease produced nonhemolytic none

+ Bryan

* U. S. Naval Medical School

TABLE II (Continued)

Characteristic	<u>Gaffkya Tetragena</u>	<u>Alpha</u>
ORIGINAL SOURCE	human skin wounds and abscesses	a specimen of solid propellant containing aluminum powder, ethylene oxide, and an inoculum of <u>B. subtilis</u> var. <u>niger</u> spores
HABITAT	skin and mucous membranes of humans and other animals, and in the air	dust at JPL - possibly also people
DISTINCTIVE CHARACTERISTICS	<p>tetrads in body fluids</p> <p>does not reduce nitrates</p> <p>growth as sediment in broth</p>	<p>appearance in FTM</p> <p>form of colonies</p> <p>frequency of tetrads in smears from surface colonies</p> <p>ethylene oxide-resistance</p> <p>sensitivity to drying from suspension in pure solvents</p>

TABLE III

GROWTH RATE OF STAPHYLOCOCCUS EPIDERMIDIS STRAINS

Tubes of Trypticase soy broth which showed turbidity after inoculation with two different strains of Staphylococcus epidermidis and incubation at 37°C for several periods of time

Length of Incubation hours	Strain ¹	Size of Inoculum ² , logarithm of one-half the estimated number of viable particles											
		6	5	4	3	2	1	0	-1	-2	-3	-4	-5
16	x	+	+	-	-	-	-	-	-	-	-	-	-
	155a			+	+	-	-	-	-	-	-	-	-
	155b			+	+	-	-	-	-	-	-	-	-
19	x	+	+	+	+	+	-	-	-	-	-	-	-
	155a			+	+	+	-	-	-	-	-	-	-
	155b			+	+	+	-	-	-	-	-	-	-
24	x	+	+	+	+	+	+	-	-	-	-	-	-
	155a			+	+	+	+	+	-	-	-	-	-
	155b			+	+	+	+	-	-	-	-	-	-
40	x	+	+	+	+	+	+	-	+	-	+	-	-
	155a			+	+	+	+	+	+	+	-	-	-
	155b			+	+	+	+	+	-	-	-	-	-
48	x	+	+	+	+	+	+	-	+	-	+	-	-
	155a			+	+	+	+	+	+	+	-	-	-
	155b			+	+	+	+	+	-	-	-	-	-

1. Strain x is a mutant of species alpha which is not resistant to ethylene oxide dissolved in broth to the extent of 0.4% and which does not liquify gelatin as alpha does. Strains 155a and 155b are replicates, of a suspension of cells in purified water, obtained from a single ampoule of ATCC 155 strain of Staphylococcus epidermidis.
2. Cells of Staphylococci occur mostly in multiples, i.e., diploids, tetrads, and small clusters (the name means cocci which occurs in grapelike clusters). For this reason, assays disclose the numbers of viable clusters or particles. On serial dilution, some of the clusters are broken into smaller clusters which extends the range of infectivity of suspensions to greater dilutions. It also means that at the higher concentrations the actual number of viable clusters is less than that indicated by conventional interpretations of serial dilution assays.

TABLE IV

TOXICITY OF SOLVENTS FOR MICROORGANISMS

Survival of microorganism populations (2×10^4 cells) in suspension in several common solvents at room temperature for two periods of time.

Suspension Vehicle	<u>B. subtilis. var. niger</u> <u>Staph. epidermidis, sp.</u>			
	0 hours	4 hours	0 hours	4 hours
Water	+	+	+	+
Ethanol 99%, water 1%	+	+	-	-
Acetone 99%, water 1%	+	+	-	-
Methanol 99%, water 1%	+	+	-	-

- One hundred thousand (10^5) cells in 0.1 ml of water were dispersed in 10 ml of the solvent. Five percent of this population, in 0.5 ml of the solvent, was transferred to Trypticase soy agar and incubated at 37°C for 3 days. The + signs indicate some of the population survived. The - signs indicate that no survivors were detected.

TABLE V

TOXICITY OF PROPELLANT INGREDIENTS

+ indicates the inoculum grew in the broth, - indicates that it did not grow.

Species	Broth	Propellant Ingredient														
		ETO ¹					Log ₁₀ of % V/V TDI					Log ₁₀ of % V/V PPG				
		1.2	0.5	-0.3	-1.0	-2.7	-∞	1.2 ³	0.5 ³	-0.3 ³	-1.0 ³	-2.7 ³	1.2 ³	0.5 ³	-0.3 ³	-1.0 ³
<u>B. subtilis</u> , var. <u>niger</u> spores	TSB ²	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
	FTM	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
<u>Staph.</u> <u>epidermidis</u> sp. x	TSB	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
	FTM	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
<u>Staph.</u> <u>epidermidis</u> ATCC 155	TSM	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
	FTM	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+

1. ETO is ethylene oxide. The concentration is expressed in terms of volume of liquid ethylene oxide

TDI is 2,4-toluene diisocyanate

PPG is polypropylene glycol

AMP is ammonium perchlorate

TSB is Trypticase soy broth

FTM is fluid thioglycollate medium

2. Into each tube containing 5 ml of the indicated broth were put 1 ml of the propellant ingredient, suspended in enough water to produce the proper concentration, and 0.1 ml of a water suspension of the microorganisms containing 10⁴ viable particles.

3. The broth was saturated with the propellant ingredient (in the case of TDI, the reaction product of TDI and water). A second phase, consisting primarily of the propellant ingredient or reaction product, was also present in the broth tube.

TABLE VI
TOXICITY OF PROPELLANT INGREDIENTS

Ingredient	Inoculum	Vigor of Microbial Growth in Broth				
		Exposed to Ethylene Oxide		No	No	Yes
		Exposed to Ammonium Perchlorate		No	Yes	Yes
Aluminum Powder (MD 105, Lot # A1163-112)	10 ⁶			+		
	10 ⁴					
	10 ²					
	0					
	10 ² A ²					+
	10 ² B ³			++		++
Alroperse (Lot # 85377)	10 ⁶					
	10 ⁴					
	10 ²					
	0					
	10 ² A ²					
	10 ² B ³					++
PBNA, Phenyl-beta-naphthylamine (Lot #54)	10 ⁶					
	10 ⁴					
	10 ²					
	0					
	10 ² A ²					
	10 ² B ³			++		++
TMP, 1, 1, 1-Trimethylol propane	10 ⁶					
	10 ⁴					
	10 ²					
	0					
	10 ² A ²					
	10 ² B ³			++	Δ+	++
FeAA, Ferric acetylacetonate (xx mod., 0.010 screen, ground 8-3-63)	10 ⁶					
	10 ⁴					
	10 ²					
	0					
	10 ² A ²					
	10 ² B ³			+		++
Acetone	10 ⁶					
	10 ⁴					
	10 ²					
	0					
	10 ² A ²					
	10 ² B ³			++		++
TDI, 2, 4-Toluene diisocyanate (Lot #279)	10 ⁶					
	10 ⁴					
	10 ²					
	0					
	10 ² A ²					
	10 ² B ³			++		++
PPG, Polypropylene glycol (BH-3-252278 DR 21 degassed 2-2-64)	10 ⁶					
	10 ⁴					
	10 ²					
	0					
	10 ² A ²					
	10 ² B ³			++		++

¹ (Lot #7883 DR 27 and 28)

² Bacteriostasis control for Alpha

³ Bacteriostasis control for B. subtilis, var. niger spores

⁴ While B. subtilis, var. niger did not grow, a contaminant did.

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TABLE VII

ETHYLENE OXIDE RESISTANCE OF SEVERAL SPECIES OF BACTERIA

+ indicates that the inoculum grew in the broth when incubated 40 hours at 37°C, - indicates that the inoculum did not grow.

Species	Inoculum, particles ³	% V/V of Liquid Ethylene Oxide ¹ in TSB ²				
		0.60	0.40	0.20	0.10	0.5 or less
<u>B. subtilis</u> , var. <u>niger</u> spores	10 ²	-	-	-	-	+
		-	-	-	-	-
	10 ⁶	-	-	+	+	+
		-	-	+	+	
<u>Staph. epidermidis</u> ATCC 155	10 ⁴	-	-	-	+	+
		-	-	-	+	+
	10 ⁵	-	-	+	+	+
		-	-	+	+	
<u>Staph. epidermidis</u> sp. x	10 ⁴	-	-	-	+	+
		-	-	+	+	+
<u>Alpha</u>	10 ⁴	-	-	-	+	+
		-	+	+	+	+
	10 ⁵	+	+	+	+	+
		-	+	+	+	
		-	+	+	+	
		-	-	+	+	

1. The ethylene oxide was contained in 1 ml of an aqueous solution.
2. TSB means Trypticase soy broth.
3. The inoculum was contained in 0.1 ml of viable particles suspended in water.

TABLE VIII

INOCULUMS OF EACH OF SEVERAL SPECIES OF MICROORGANISMS REQUIRED TO INFECT FLUID THIOGLYCOLLATE MEDIUM CONTAINING ETHYLENE OXIDE
 Number of + signs indicates density of turbidity. - signs indicate no turbidity appeared.

Species	Inoculum Cells	Percent of Ethylene Oxide in Fluid Thioglycollate Medium			
		0.00	0.38	0.70	0.96
Bacillus from ethylene oxide-exposed SY 251/7 propellant specimen, species <u>Zavin</u> from Task II.	3×10^7	---	---	---	---
	3×10^6	+++	+++	+++	+++
	3×10^5	+++	+++	++	++
	3×10^4	+++	+++	++	++
	3×10^3	+++	+++	-	-
	3×10^2	+++	+++	++	-
<u>B. lichenformis</u>	1×10^7	---	---	---	---
	1×10^6	+++	++	++	-
	1×10^5	+++	++	-	-
	1×10^4	+++	++	-	-
	1×10^3	+++	-	-	-
	1×10^2	---	---	---	---
<u>Beta</u>	1×10^7	---	---	---	---
	1×10^6	+++	+++	+++	-
	1×10^5	+++	+++	+++	+++
	1×10^4	+++	+++	+++	+++
	1×10^3	+++	+++	-	-
	1×10^2	+++	-	-	-
<u>B. subtilis</u> , var. <u>niger</u>	3×10^7	+++	+++	-	-
	3×10^6	+++	+++	-	-
	3×10^5	+++	++	-	-
	3×10^4	+++	++	-	-
	3×10^3	+++	-	-	-
	3×10^2	---	---	---	---
<u>Alpha</u>	2×10^7	+++	+++	+++	+++
	2×10^6	+++	+++	+++	+++
	2×10^5	+++	+++	+++	+++
	2×10^4	+++	+++	+++	+++
	2×10^3	+++	+++	+++	++
	2×10^2	---	---	---	---
<u>Staphylococcus epidermidis</u>	1×10^7	---	---	---	---
	1×10^6	+++	++	+	+
	1×10^5	+++	++	++	+
	1×10^4	+++	++	+	+
	1×10^3	+++	++	+	+
	1×10^2	+++	++	+	-

TABLE IX
EFFECT OF DRYING ON VIABILITY OF
STAPHYLOCOCCUS EPIDERMIDIS POPULATIONS

Numbers of colonies on Trypticase soy agar incubated at 37°C

Inoculum ¹	Expected Number of Colonies	Strain						
		ATCC 155		Alpha				
		Test 1	Test 2	Test 1	Test 2	Test 3	Test 4	Test 5
10 ⁸	2,000,000				320			
10 ⁷	200,000		496	4	50			TNC
10 ⁶	20,000	1000	7	1	1	TNC	126	
10 ⁵	2,000	260	7	0	0	20	80	
10 ⁴	200	0	0	0	0	1	4	
10 ³	20	0		0		0	0	

1. The inoculum was contained in 0.1 ml of a liquid suspension of the cells. It was dried on a glass cover slip in an open Petri dish for a period of 4 hours. The inoculum remained saturated for two of these four hours. In both Tests 1 and 2 for both Strains, the cells had been washed before they were suspended in sterile purified water and used to inoculate the glass cover slips. In Tests 1 and 2 the inoculum was dried in air at 50% RH. The cells used in Tests 3, 4, and 5 had not been washed. For Tests 3 and 4 the inoculums represent a serial dilution of material flushed from a partially dried culture on the surface of Trypticase soy agar. Though the dilutions were made in sterile purified water, some of the agar and nutrients surely accompanied the cells into the inoculum where they could protect the cells from destruction by drying. In Test 5 the inoculum was from a three-day-old culture in fluid thioglycollate medium. While the inoculum in Test 5 was dried simultaneously with and similarly to that of Test 2, the inoculum for Test 3 was dried in dry air and for Test 4 in dry nitrogen.

TABLE X
EFFECTS OF DRYING AND EXPOSURE OF ETHYLENE OXIDE
ON POPULATIONS OF ALPHA ORGANISMS

Number¹ of colonies on each Trypticase soy agar plate after inoculation with a 0.5-ml aliquot of the fluid thioglycollate suspension² of the dried³ inoculum.

Support	Desiccant	Replicate	Exposed ⁴ to Ethylene Oxide Vapor				Not Exposed to Ethylene Oxide Vapor			
			In Acetone		In Water		In Acetone		In Water	
			Alpha	Spores	Alpha	Spores	Alpha	Spores	Alpha	Spores
Glass Coupon, 1" x 1" x 0.05"	Dry Nitrogen	1		0				5		60
		2		0				0		100
	Dry Air	1	0			0		6	0	
		2	0			0		0	0	
	Air at 50% RH	1	0			0		10	0	
		2	0			0		42	0	
Teflon Coupon, 1" x 1" x 0.01"	Dry Nitrogen	1	0			0		0	0	
		2	0			0		0	0	
	Dry Air	1	0			0		1	0	
		2	0			1		1	0	
	Air at 50% RH	1		0	0		0			62
		2		2	0		0			70
Aluminum Coupon, 1" x 0.03"	Dry Nitrogen	1	0					19	0	
		2	0					24	0	
	Dry Air	1		0	0		0			28
		2		2	0		0			25
	Air at 50% RH	1	0					15	0	
		2	0					5	0	

- Expected number of colonies, had all viable particles in the inoculum on the coupon survived drying and had been evenly dispersed in the fluid thioglycollate medium, was 2×10^4 . The inoculum on the coupon contained, initially, 10^6 particles in 0.1 ml of liquid. On drying these particles could form clumps which were not dispersed adequately enough to disclose the true numbers of survivors.
- The dried inoculum was suspended in previously sterile fluid thioglycollate medium by putting the coupon (glass, teflon, or aluminum) into the medium aseptically and then sonicating the medium for 1 minute before shaking the jar of medium and withdrawing the 0.5-ml aliquot. The coupons were all prepared and tested in duplicate.
- The inoculum included 10^6 viable particles (alpha occurs mostly as diploids, tetrads, and small groups of cells) dried for 24 hours in the indicated environments.
- The exposure was to saturated ethylene oxide vapor, containing water vapor in excess of that equivalent to 50% RH and a small amount of air. The exposure took place at room temperature. Exposure lasted for 2 hours.

TABLE XI

EFFECT OF DRYING AND EXPOSURE TO ETHYLENE OXIDE ON
DETECTABILITY OF POPULATIONS OF ALPHA ORGANISMS¹

The number of + signs indicates the turbidity of the fluid thioglycollate medium containing the dried inoculum and coupon after incubation, - signs indicate that no turbidity appeared.

Support	Desiccant	Replicate	Exposed to Ethylene Oxide Vapor				Not Exposed to Ethylene Oxide Vapor			
			In Acetone		In Water		In Acetone		In Water	
			Alpha	Spores	Alpha	Spores	Alpha	Spores	Alpha	Spore
Glass	Dry Nitrogen	1		-	++			+++		+++
		2		-	-			+		+++
	Dry Air	1	+++			-		+++	-	
		2	+++			-		+++	+++	
	Air at 50% RH	1	-			++		+++	-	
		2	-			-		+++	+++	
Teflon	Dry Nitrogen	1	-			+		+++	+	
		2	-			+++		+++	+	
	Dry Air	1	-			+++		-	+	
		2	-			-		+++	-	
	Air at 50% RH	1		-	-	-	-			+++
		2		++	-	-	+			+++
Aluminum	Dry Nitrogen	1	-					+++	-	
		2	-					+++	+++	
	Dry Air	1		-	-		+			+++
		2		-	-		+			+++
	Air at 50% RH	1	+++			-		+++	+	
		2	-			-		+++	+	

1. The populations and their treatments are described in the footnotes to Table X.

TABLE XII

COLOR¹ OF FLUID THIOLGYCOLLATE MEDIUM CONTAINING
DRIED INOCULUM AND COUPON AFTER INCUBATION²

Support	Desiccant	Replicate	Exposed to Ethylene Oxide Vapor				Not Exposed to Ethylene Oxide Vapor			
			In Acetone		In Water		In Acetone		In Water	
			Alpha	Spores	Alpha	Spores	Alpha	Spores	Alpha	Spores
Glass	Dry Nitrogen	1		straw	yellow			yellow		yellow
		2		straw	amber			amber		yellow
	Dry Air	1	yellow			straw		yellow	amber	
		2	yellow			straw		yellow	amber	
	Air at 50% RH	1	amber			amber		yellow	amber	
		2	amber			amber		yellow	amber	
Teflon	Dry Nitrogen	1	straw			amber		yellow	amber	
		2	straw			yellow		straw	amber	
	Dry Air	1	straw			yellow		yellow	amber	
		2	straw			straw		yellow	amber	
	Air at 50% RH	1		amber	amber		amber			yellow
		2		amber	amber		amber			yellow
Aluminum	Dry Nitrogen	1	yellow					yellow	amber	
		2	straw					yellow	amber	
	Dry Air	1		straw	straw		amber			yellow
		2		straw	straw		amber			yellow
	Air at 50% RH	1	straw			amber		yellow	amber	
		2	straw			amber		yellow	amber	

1. A 20-ml quantity of freshly prepared fluid thioglycollate medium in baby-food jars is straw colored. Because the head space over the medium contains oxygen, the broth soon becomes saturated and amber in color, but remains free of turbidity. Characteristically, the medium becomes increasingly reducing as populations of microorganism grow and consume oxygen. The amber color then disappears and a yellow color develops. The yellow color is usually accompanied by a dense turbidity. Occasionally the microorganism population does not become large enough to produce turbidity but the broth develops a straw color again, indicating oxygen consumption.
2. The broth had been sampled to prepare the agar plates described in Table X. Moreover, some coupons contained viable particles of species other than alpha or B. subtilis, var. niger. These particles produced yellow color in the broth. In interpreting the table, "amber" means that the broth appeared to contain no viable nor metabolizing cells from the inoculum. All amber colored broths were confirmed to be nonbacteriostatic.

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MICROORGANISMS IN SOLID MATERIALS

TASK II:

NATURALLY OCCURRING MICROBIOLOGICAL FLORA
FROM NORMALLY PREPARED PROPELLANT SPECIMENS

(Mod. 1)

VOLUME IV

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SUMMARY

The extent and character of microbiological contamination in each of two batches of solid propellant were measured. In a portion of one batch a population as large as 10^6 cells/milliliter was found. While no thermophiles nor obligate anaerobes were detected, many species of aerobes and facultative anaerobes were. Several of these species appear to be more resistant to ethylene oxide than are spores of B. subtilis, var. niger.

I. INTRODUCTION

That solid propellants may contain microorganisms more resistant to ethylene oxide than are spores of B. subtilis var. niger was indicated by discovery of species of alpha. Species alpha was found in aerobic incubation of pulverized solid propellant dispersed in Trypticase soy broth and incubated at 37°C. This propellant specimen had been prepared from a formulation which had included both ethylene oxide and an inoculum of B. subtilis var. niger spores. Whether normally prepared solid propellant might also contain anaerobes, thermophiles or molds more resistant than either alpha or B. subtilis var. niger, when embedded in solid propellant, had been unresolved.

Replicated specimens of two types of solid propellant and of a Pfaudler-pot fuel mix (containing Alrospense, polypropylene glycol, and 1, 1, 1 - trimethylol propane) were assayed for viable cell content and the ethylene oxide resistant species found identified with respect to morphology, Gram-staining characteristics, and colony form and color.

One specimen (SY 251/7) contained aluminum particles while the other (PUP 2/11) did not. Both propellants use a polyurethane-type binder. Alpha was isolated originally from a propellant similar to SY 251/7.

II. METHODS

The solid propellant specimens identified in Table I were assembled in hermetically-sealed, polyethylene bags with the tools and supplies shown in Figure 1. The specimens were in blocks 2-1/2" x 3" x 3" in size. The polyethylene bag was constructed of 4-mil polyethylene film and the seams were sealed by a heat sealing process. In each bag with the propellant specimen and supplies, was placed a jar containing a solution consisting of 100 milliliters of liquid ethylene oxide and 1 milliliter of water. This solution evaporated to saturate the air in the bag with ethylene oxide vapor and maintain the absolute humidity in the bag about that equivalent to 50 percent relative humidity. The concentrations of ethylene oxide and water in the bag were confirmed by gas chromatography. The bags remained sealed for a period of 72 hours before the solid propellant specimens were sampled and pulverized particles dispersed in the previously sterile fluid thioglycollate medium (FTM) in baby food jars.

To sample a solid propellant specimen, it was transferred to one of the two powder collection compartments where it was abraded by the file while dry. All particles were less than one millimeter in greatest dimension with many of them in the micron range of sizes. The specimen and the file were removed from the powder collection compartment and were replaced by a jar containing 100 milliliters of sterile fluid thioglycollate broth. During the surface sterilization process the exterior of the specimen had maximum exposure to ethylene oxide. If ethylene oxide penetrated the specimen, the exterior portion would show maximum effect of this penetration. In Table I the viable particle content of this powder is shown for those specimens which have been identified as "exterior (ETO)".

After approximately one gram of the powder was prepared and the replacement made, the passage between the compartment and the remainder of the bag was then closed using a heat sealer.

The contents of the jar of broth were poured into the hermetically sealed powder collection compartment. After verifying the integrity of the seal, the entire bag and its contents were sonicated for one minute. The broth, now containing the pulverized propellant, was returned to the jar which was then sealed and removed from the plastic envelope. The jar was wiped dry.

Previous abrasion techniques have been performed while the specimen was submerged in broth or water in order to suppress the fire hazard. Under aqueous liquids, the ammonium perchlorate is rapidly extracted from the propellant and the abrasion occludes many organisms while it exposes others. When pulverized in the dry state, the structure of the propellant is preserved during the wetting with broth or water thereby reducing occlusion of viable cells.

On wetting, the powdered propellant readily agglomerates into a clump. On working, this clump can be compacted easily. In testing for viable cells, avoidance of compaction is important and for this reason, the suspension including the clump was sonicated to hasten the penetration of the broth and the solution of the ammonium perchlorate.

After the exterior sample had been removed from the specimen, the specimen was sliced along the dotted lines shown in Figure 1. This cut exposed an interior surface for sampling. The smaller portion of the specimen was then put into the second powder collection compartment and approximately one gram of powder abraded from the freshly cut "interior" surface. The steps for suspending the powder in the broth were repeated.

The gas remaining in the bag with the tools and two blocks of specimen was then assayed for ethylene oxide and water vapor contents. The ethylene oxide content, in every case, was found to be less than 70 milligrams per liter and the water vapor content to exceed that equivalent to 50 percent relative humidity.

The bag for one of the propellant specimens contained, in addition to the tools and supplies discussed above, a microtome, tweezers, and a third jar of broth. After the powder specimens had been collected, a

portion of propellant from the interior of the specimen was placed in the microtome holder and slices approximately 400 microns thick were removed from it and placed in the previously sterile broth. Approximately one gram of propellant was transferred to the broth in this manner.

Using a sterile pipette and aseptic technique, one milliliter of the Pfaudler-pot fuel mix was transferred to the previously sterile broth.

Bacteriostasis controls on the broth were prepared by having a third bottle of broth in two of the bags. These broths were confirmed to be nonbacteriostatic to spores of Cl. sporogenes. After 72 hours incubation, all jars still showing no evidence of bacterial populations were inoculated with one-half milliliter of the fluid from one of the jars showing turbidity. On further incubation, turbidity and a yellow color developed in every one of the jars, verifying absence of strong bacteriostatic effects.

Within two hours after the pulverized specimen was suspended in the previously sterile fluid thioglycollate medium (FTM), a 0.5-milliliter aliquot of the suspension was withdrawn and placed on the surface of Trypticase soy agar (TSA) and incubated there under each of the conditions shown in Table I. In addition, Sabourauds dextrose agar (Sab) and anaerobic agar (AA) in large, deep test tubes were inoculated with 0.5-milliliter aliquots of the suspension and incubated under each of the corresponding conditions shown in Table I.

The jars containing the suspension of pulverized propellant and the Pfaudler-pot fuel mix were incubated at 37°C for 6 hours (10 hours in the case of Specimens No. 4 and 6) after which a second set of plates and AA tubes were inoculated with 0.5-milliliter aliquots. The jars were then returned to the incubator where they remained until a total of 72 hours had elapsed. They were then sampled again for the presence of anaerobes and then tested for bacteriostasis in the manner described above.

All plates were incubated for 7 days before final colony counts were recorded. On the right side of Table I, the information under the heading, "in FTM at 37" describes the appearance of the broth in the baby food jars when they were tested for the presence of anaerobes and bacteriostasis.

Under the heading "on Brewer plates" are recorded the numbers of colonies appearing on the surface of anaerobic agar (AA) inoculated with 0.5 milliliters of the fluid in the incubated baby food jars. Anaerobic incubation was provided by use of Brewer anaerobic plates. In most cases the colonies were mucoid and coalesced to form one large colony.

The integrity of the same closure on these same baby food jars, with respect to ethylene oxide penetration, was established by many tests in earlier laboratory studies.

The footnotes following Table II present specific information about the details of the tests whose results are reported in that table.

III. RESULTS

Table I shows the numbers of naturally occurring microorganisms which can be detected in five milligrams of pulverized solid propellant or Pfaudler-pot fuel mix. Two of the specimens contained in excess of 10^6 viable cells per milliliter. The most prevalent species seemed to be a Gram-negative aerobic bacillus which was not, however, particularly resistant to ethylene oxide in ethylene oxide-screening culture but seemed to survive exposure to surface sterilization. The preparation of the ethylene oxide-screening culture is described in a note following Table II.

On incubation of the suspension of pulverized propellant, the numbers of viable cells of some species seemed to decrease while those of other species seemed to increase. The frequency of occurrence of cells of anaerobic species seemed to parallel the frequency of appearance of cells of aerobic species.

When the plates contain many colonies, the frequency of colonies from two or more viable cells coalescing to appear as one colony increases. In addition, cells which are late in germinating find that the agar has been depleted of nutrients by those cells which developed colonies earlier. Some strains and species produce substances which suppress the proliferation of other strains of microorganisms. In the interpretation of the information in Table I, little reliance can be placed on the numbers recorded for a particular type of colony when either many colonies or many types of colonies were observed. The information in the table does show that never more than five really distinct types of colonies appeared on any one plate and that some plates contained relatively many colonies while other plates contained relatively few. Comparisons of relative numbers of colonies of different species appearing on any particular plate are not meaningful. Differences may be due more to conditions of incubation than anything else.

Earlier work had found that as few as 100 viable spores per milliliter of B. subtilis var. niger in solid propellant, which contained aluminum, were detectable when the propellant was pulverized by shaving with a microtome. No B. subtilis var. niger cells were found in any of the propellant studied in the program reported here, though cells resembling species alpha were found. A variety of reasons why the microtomed specimen failed to show contamination could be offered, but to establish which reasons are correct would have required an effort beyond the scope of the program. It was sufficient to show that the file abrasion of dry propellant was a sufficiently sensitive technique for detection of microbial contamination to be useful.

Table I shows that the microbial contamination was not uniform throughout a single block of cast propellant. This observation may indicate that the microbial contamination was introduced late in the mixing process or during casting. This indication is supported by the failure of the testing technique used to find any viable cells in the Pfaudler-pot fuel mix or any of the ingredients of the propellants. These techniques were not sensitive enough to measure very low orders of contamination but were established to measure moderately large contamination loads.

Table II describes the characteristics of many of the species of microorganisms found in the solid propellant specimens. The characteristics of the species alpha, found earlier in solid propellant, beta, found to be a particularly ethylene oxide-resistant species present in dust at JPL, and phi, identified as a strain of B. lichenformis, have been included in the table. For purposes of colony description the facultative species, other than alpha were grown on anaerobic agar in Brewer anaerobic plates and in fluid thioglycollate medium rather than on the Trypticase soy substrates. The descriptions of colonies on the anaerobic plates may not correspond with those of ethylene-oxide resistance because the anaerobic plates contained mixed cultures of possibly widely varying resistance to ethylene oxide.

By the Greek and Hebrew letters in front of the numbers, Table I shows the source of many of the species of microorganisms described in Table II. The five colony types indicated in Table I do not signify the same types of colonies for all of the plates. The presence of other species and

greater numbers of colonies on some plates than on others causes a change in the sizes and appearances of the colonies. In addition, the appearance of the colonies of a particular species will vary with incubation conditions. For this reason, the set of colonies chosen for identification may contain replications and most likely will.

IV. CONCLUSIONS

1. Several varieties of microorganisms are present in the solid propellant, some to the extent of 10^6 per milliliter. Most of the easily detected species are aerobes though many facultative anaerobes are also present. Thermophiles are few.
2. Among the species of microorganisms found in the solid propellant were several more resistant to ethylene oxide than are spores of B. subtilis var. niger under the conditions of the test.
3. The extent of contamination varies within a single block of cast propellant. The greatest contamination is not necessarily either the first or last portion cast.
4. The microbial contamination in the specimens containing aluminum powder (SY 251/7) produced fewer colonies on the assay plates than did the specimens which contained no aluminum powder. While this effect could have been due to the presence of the aluminum particles, it could equally well be due to the batch-to-batch variation in the preparation of the propellant. The absence of microbial flora in the pot fuel mix indicates that detectable microorganisms were present in numbers fewer than 10 per milliliter on the basis of the sampling statistics. If the pot fuel mix is bacteriostatic to any degree even 10 cells/milliliter may fail to show their viability in the assay procedures used. The difference in numbers of viable microorganisms in the propellant and in the Pfaudler-pot fuel mix indicates that the contamination occurred after this fuel mix was prepared. The work in Task I indicates also that the contamination came either from accidental contamination during the mixing or casting operation or that microorganisms of certain species proliferate in the solid propellant. The specimen which contained the greatest number of viable cells was stored for 8 months before it was sampled and tested for microorganism content.

5. On suspension of microorganisms in broth, the numbers of some species decrease so that the ability to detect the presence of these species depends upon the promptness of the assay of the suspension.
6. Abrasion of the propellant while it is dry produces fine particles from which microorganisms can be recovered at least as well as from microtomed slices 400 microns thick.
7. Under the conditions used, ethylene oxide does not penetrate a block of solid propellant deeply. Some does penetrate deeply enough, however, to reduce the size of the viable population near the surface.

TABLE I

NATURALLY OCCURRING POPULATIONS OF MICROORGANISMS IN
NORMALLY PREPARED SOLID PROPELLANT

Numbers of Each of Several Types of Colonies

Specimen	Plate	Colony Type	A 0.5-ml Aliquot of the Suspension ¹ of Pulverized Specimen Was Incubated		A 0.5-ml Aliquot of the Suspension of Pulverized Specimen after Six Hours ² at 37 deg. C was Incubated				
			Aerobically		Anaerobically	Aerobically			
			on TSA at 55°	on TSA at 37°	on TSA at 25°	on TSA at 25°	on TSA at 37°	on TSA at 25°	Anaerobically in FTM at 37°
1. From interior of a 3"x3" x 2 1/2" block cut from top of a larger block of propellant identified as PUP 2/11	a	1	0	19	29	11	0	0	21
		2	0	~ 1500	~ 400	0	~ 1000	0	0
		3	0	0	0	0	0	35	0
		4	0	~ 100	~ 200	~ 200	0	0	0
		5	0	0	0	0	0	0	0
2. From interior of a 3"x3" x 2 1/2" block cut from bottom of a larger block of propellant identified as PUP 2/11	b	1	0	28	25	8	0	~ 200	38
		2	0	~ 1500	~ 400	0	~ 1000	0	0
		3	0	0	0	0	0	25	0
		4	0	~ 100	~ 200	~ 200	0	0	0
		5	0	0	0	0	0	0	0
3. From interior of a 3"x3" x 2 1/2" block cut from bottom of a larger block of propellant identified as PUP 2/11	a	1	0	19	29	20	0	Overgrown by colonies of type number 1	0
		2	0	~ 1500	~ 500	80	~ 1000	0	10
		3	0	0	0	0	0	0	0
		4	0	~ 200	~ 150	100	0	?	0
		5	0	0	0	0	0	0	0
4. From interior of a 3"x3" x 2 1/2" block cut from bottom of a larger block of propellant identified as PUP 2/11	b	1	0	32	48	30	0	~ 2000	0
		2	0	~ 1500	~ 500	15	~ 1000	~ 2000	12
		3	0	0	0	0	0	0	0
		4	0	~ 200	~ 100	~ 100	0	?	0
		5	0	0	0	0	0	0	0

¹One gram (approximately) of the pulverized propellant specimen (or other material) was suspended in 100 ml of fluid thioglycollate medium in a baby food jar.

²The pulverized specimen in the fluid thioglycollate medium was stored for six hours at 37°C before a second 0.5 ml of it was put on each of the indicated substrates.

TABLE I (Continued)

Numbers of Each of Several Types of Colonies

Specimen	Plate	Colony Type	A 0.5-ml Aliquot of the Suspension ¹ of Pulverized Specimen Was Incubated										A 0.5-ml Aliquot of the Suspension of Pulverized Specimen after Six Hours ² at 37 deg. C was Incubated									
			Aerobically					Anaerobically					Aerobically					Anaerobically				
			on TSA at 55°	on TSA at 37°	on TSA at 25°	on Sab at 25°	in AA at 37°	on TSA at 55°	on TSA at 37°	on TSA at 25°	Overgrown with colonies of type number 1	on Sab at 25°	in AA at 37°	on TSA at 55°	on TSA at 37°	on TSA at 25°	in AA at 37°	in FTM at 37°	on Brewer Plates			
3. From interior of a 3" x 3" x 2 1/2" block cut from top of a larger block of propellant identified as SY 251/7	a	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	yellow brown and turbid	overgrown with confluent colonies				
		2	0	7	0	3	0	0	0	1	0	0	0	0	0	0	0	0				
		3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
		4	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0				
		5	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
	b	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0						
		2	0	7	0	2	0	0	0	0	0	0	0	0	0	0	0	0				
		3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
		4	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0				
		5	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0				
4. From interior of a 3" x 3" x 2 1/2" block cut from bottom of a block of propellant identified as SY 251/7	a	1	0	0	1	1	1	1	0	0	0	0	0	0	0	0	yellow and turbid broth	overgrown with confluent colonies				
		2	0	2	0	25	~2000	~1000	~1000	0	0	0	0	0	0	0	0	0				
		3	0	160	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
		4	0	0	0	25	0	0	0	0	0	0	0	0	0	0	0	0				
		5	0	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0				
	b	1	0	0	0	1	0	0	0	~1000	0	0	0	0	0	0						
		2	0	90	0	25	~2000	~1000	~1000	0	0	0	0	0	0	0	0	0				
		3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
		4	0	0	70	60	0	0	0	0	0	0	0	0	0	0	0	0				
		5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				

¹One gram (approximately) of the pulverized propellant specimen (or other material) was suspended in 100 ml of fluid thioglycollate medium in a baby food jar.

²The pulverized specimen in the fluid thioglycollate medium was stored for six hours at 37°C before a second 0.5 ml of it was put on each of the indicated substrates. The suspension of the "bottom" specimen was at 37°C for 10 hours before second aliquot was taken.

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TABLE I (Continued)

Numbers of Each of Several Types of Colonies

Specimen	Plate	Colony Type	A 0.5-ml Aliquot of the Suspension ¹ of Pulverized Specimen Was Incubated					A 0.5-ml Aliquot of the Suspension of Pulverized Specimen after Six Hours ² at 37 deg. C was Incubated				
			Aerobically					Anaerobically				
			on TSA at 55°	on TSA at 37°	on TSA at 25°	on TSA at 25°	on TSA at 37°	on TSA at 55°	on TSA at 37°	on TSA at 25°	in AA at 37°	in FTM at 37°
5. From exterior (ETO) of a 3"x3" x 2 1/2" block cut from top of a larger block of propellant identified as SY 251/7	a	1	0	0	0	0	0	0	0	0	0	0
		2	0	0	0	0	0	0	0	0	0	0
		3	0	0	0	0	0	0	0	0	0	0
		4	0	0	0	0	0	0	0	0	0	0
		5	0	0	0	0	0	0	0	0	0	0
6. From exterior (ETO) of a 3"x3" x 2 1/2" block cut from bottom of a larger block of propellant identified as SY 251/7	b	1	0	0	0	0	0	0	0	0	0	0
		2	0	0	0	0	0	0	0	0	0	0
		3	0	0	0	0	0	0	0	0	0	0
		4	0	0	0	0	0	0	0	0	0	0
		5	0	1	0	0	0	0	0	0	0	0
6. From exterior (ETO) of a 3"x3" x 2 1/2" block cut from bottom of a larger block of propellant identified as SY 251/7	a	1	0	0	0	0	0	0	0	0	0	0
		2	0	0	0	0	0	0	0	0	0	0
		3	0	0	0	0	0	0	0	0	0	0
		4	0	0	0	0	0	0	0	0	0	0
		5	0	0	0	0	0	0	0	0	0	0
6. From exterior (ETO) of a 3"x3" x 2 1/2" block cut from bottom of a larger block of propellant identified as SY 251/7	b	1	0	0	0	0	0	0	0	0	0	0
		2	0	0	0	0	0	0	0	0	0	0
		3	0	0	0	0	0	0	0	0	0	0
		4	0	0	0	0	0	0	0	0	0	0
		5	0	0	0	0	0	0	0	0	0	0

¹One gram (approximately) of the pulverized propellant specimen (or other material) was suspended in 100 ml of fluid thioglycollate medium in a baby food jar.

²The pulverized specimen in the fluid thioglycollate medium was stored for six hours at 37°C before a second 0.5 ml of it was put on each of the indicated substrates. The suspension of the "bottom" specimen was at 37°C for 10 hours before the second aliquot was taken.

TABLE I (Continued)

Numbers of Each of Several Types of Colonies

Specimen	Plate	Colony Type	A 0.5-ml Aliquot of the Suspension ¹ of Pulverized Specimen Was Incubated					A 0.5-ml Aliquot of the Suspension of Pulverized Specimen after Six Hours ² at 37 deg. C was Incubated				
			Aerobically					Anaerobically				
			on TSA at 55°	on TSA at 37°	on TSA at 25°	on Sab at 25°	in AA at 37°	on TSA at 55°	on TSA at 37°	on TSA at 25°	in AA at 37°	in FTM at 37°
From exterior (ETO) of a 3"x3"x 1/2" block cut from top of a larger block of propellant identified as PUP 2/11	a	1	0	9	8	12	0	0	51	-	45	yellow
		2	0	~ 600	~ 400	3	~ 500	0	~ 500	-	40	and
		3	0	1	0	0	0	0	7	-	0	turbid
		4	0	60	0 ~ 100	~ 100	0	0	~ 50	-	40	broth
		5	0	0	0	0	0	0	0	-	0	overgrown with confluent colonies
From exterior (ETO) of a 3"x3"x 2 1/2" block cut from bottom of a larger block of propellant identified as PUP 2/11	b	1	0	10	11	5	0	0	22	-	40	
		2	0	~ 600	~ 400	2	~ 500	0	~ 500	-	35	
		3	0	0	0	0	0	0	4	-	0	
		4	0	50	~ 100	~ 100	0	0	~ 50	-	40	
		5	0	0	0	0	0	0	0	-	0	
From exterior (ETO) of a 3"x3"x 2 1/2" block cut from bottom of a larger block of propellant identified as PUP 2/11	a	1	0	1	0	0	0	0	22	-	8	yellow
		2	0	2	0	3	30	0	7	-	3	and
		3	0	0	0	0	0	0	0	-	0	turbid
		4	0	0	2	0	0	0	0	-	0	broth
		5	0	0	0	0	0	0	0	-	2	overgrown with confluent colonies
From exterior (ETO) of a 3"x3"x 2 1/2" block cut from bottom of a larger block of propellant identified as PUP 2/11	b	1	0	0	0	0	0	0	20	-	9	
		2	0	5	0	1	20	0	3	-	4	
		3	0	0	0	0	0	0	0	-	0	
		4	0	0	10	0	0	0	0	-	0	
		5	0	0	0	0	0	0	0	-	0	

¹One gram (approximately) of the pulverized propellant specimen (or other material) was suspended in 100 ml of fluid thioglycollate medium in a baby food jar.

²The pulverized specimen in the fluid thioglycollate medium was stored for six hours at 37°C before a second 0.5 ml of it was put on each of the indicated substrates.

TABLE I (Continued)

Numbers of Each of Several Types of Colonies

Specimen	Plate	Colony Type	A 0.5-ml Aliquot of the Suspension ¹ of Pulverized Specimen Was Incubated					A 0.5-ml Aliquot of the Suspension of Pulverized Specimen after Six Hours ² at 37 deg. C was Incubated						
			Aerobically				Anaero- bically	Aerobically				Anaerobically		
			on TSA at 55°	on TSA at 37°	on TSA at 25°	on Sab at 25°	In AA at 37°	on TSA at 55°	on TSA at 37°	on TSA at 25°	on Sab at 25°	in AA at 37°	in FTM at 37°	on Brewer Plates
9. From a bottle of Pfaucler- pot fuel mix (a mix- ture of Alroperse, PPG, and TMP) identified as SY 239- 240-241	a	1	0	0	0	0	0	0	0	-	0	0	yellow	overgrown
		2	0	0	0	0	0	0	0	0	0	0	brown	with
		3	0	0	0	0	0	0	0	0	0	0	and	confluent
		4	0	0	0	0	0	0	0	0	0	0	turbid	colonies
		5	0	0	0	0	0	0	0	0	0	0		
10. From a bottle of Pfaucler- pot fuel mix (a mix- ture of Alroperse, PPG, and TMP) identified as SY 245- 246-247	b	1	0	0	0	0	0	0	0	-	0	0	yellow	overgrown
		2	0	0	0	0	0	0	0	0	0	0	brown	with
		3	0	0	0	0	0	0	0	0	0	0	and	confluent
		4	0	0	0	0	0	0	0	0	0	0	turbid	colonies
		5	0	0	0	0	0	0	0	0	0	0		

¹One gram (approximately) of the pulverized propellant specimen (or other material) was suspended in 100 ml of fluid thioglycollate medium in a baby food jar.

²The pulverized specimen in the fluid thioglycollate medium was stored for six hours at 37°C before a second 0.5 ml of it was put on each of the indicated substrates.

TABLE I (Continued)

Numbers of Each of Several Types of Colonies

Specimen	Plate	Colony Type	A 0.5-ml Aliquot of the Suspension ¹ of Pulverized Specimen Was Incubated					A 0.5-ml Aliquot of the Suspension of Pulverized Specimen after Six Hours ² at 37 deg. C was Incubated						
			Aerobically			Anaerobically	Aerobically			Anaerobically				
			on TSA at 55°	on TSA at 37°	on TSA at 25°		on Sab at 25°	in AA at 37°	on TSA at 55°	on TSA at 37°	on TSA at 25°	on Sab at 25°	in AA at 37°	in FTM at 37°
1. from interior of a 3"x3" x 2 1/2" block cut from bottom of a larger block ^b	a	1	0	0	0	0	0	0	-	0	0	0	yellow-brown and turbid	overgrown with confluent colonies
		2	0	0	0	0	0	0	-	0	0	0		
		3	0	0	0	0	0	0	-	0	0	0		
		4	0	0	0	0	0	0	-	0	0	0		
		5	0	0	0	0	0	0	-	0	0	0		
2. of propellant identified as PUP 2/11. Microtome slices were cultured	b	1	0	0	0	0	0	0	-	0	0	0		
		2	0	0	0	0	0	0	-	0	0	0		
		3	0	0	0	0	0	0	-	0	0	0		
		4	0	0	0	0	0	0	-	0	0	0		
		5	0	0	0	0	0	0	-	0	0	0		

¹One gram (approximately) of the pulverized propellant specimen (or other material) was suspended in 100 ml of fluid thioglycollate medium in a baby food jar.

²The pulverized specimen in the fluid thioglycollate medium was stored for six hours at 37°C before a second 0.5 ml of it was put on each of the indicated substrates.

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TABLE II
CHARACTERISTICS OF SPECIES OF MICROORGANISMS
RECOVERED FROM SOLID PROPELLANT SPECIMENS

Characteristics	Species Code		
	Alpha	Beta	Theta
CELLS Type Cell diameter, microns Cell length, microns Gram-staining characteristics Spore-staining characteristics Aggregation	coccus 0.5 -- Gram-positive negative tetrads and diploids	rod 1.0 5 - 6 Gram-positive granules central spores chains	rod 0.9 3 - 4 Gram-variable terminal granules central spores chains
CULTURES Oxygen requirements Ethylene oxide resistance ¹ Environmental requirements ² TSA at 55°C TSA at 37°C TSA at 25°C Sab at 25°C AA at 37°C Populations distribution in broth ³ : Uniform Sediment Clumps of filaments Pellicle Location of pellicle Clear zone at top Turbidity of culture in broth	facultative +++ - +++ ++ yes yes in FTM no -- in FTM medium	aerobe +++ +++ yes yes no yes top no dense	aerobe +++ - +++ + ++ yes yes no yes top no medium
COLONIES Colony type on agar Opacity Color Diameter, millimeters Edge Texture Profile	smooth translucent gray-white 1-3 round shiny convex	rough opaque light yellow 5 lobed dull convex	rough opaque gray-white 10 lobed dull flat

TABLE II (Continued)

Characteristics	Species Code		
	Iota	Kappa	Lambda
CELLS			
Type	rod	rod	rod
Cell diameter, microns	0.5	0.9	0.6
Cell length, microns	1 - 2	3 - 4	3
Gram-staining			
Characteristics	negative	Gram-positive	Gram-positive
Spore-staining			
Characteristics	negative	terminal spores	negative
Aggregation	clusters	single, diploid	clusters
CULTURES			
Oxygen requirements	aerobe	aerobe	aerobe
Ethylene oxide resistance ¹	-	+	+++
Environmental requirements ²			
TSA at 55°C	-	-	-
TSA at 37°C	+++	+++	+++
TSA at 25°C	++	++	
Sab at 25°C	+	+	
AA at 37°C			
Populations distribution in broth ³ :			
Uniform	---	yes	yes
Sediment	---	yes	yes
Clumps or filaments	---	no	no
Pellicle	---	no	no
Location of pellicle	---	---	---
Clear zone at top	---	no	no
Turbidity of culture in broth	---	light	dense
COLONIES			
Colony type on agar	smooth	smooth	rough
Opacity	transparent	translucent	opaque
Color	light yellow	orange	gray-white
Diameter, millimeters	1/2	3	2
Edge	round	lobed	irregular
Texture	shiny	shiny	dull
Profile	convex	flat	flat

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TABLE II (Continued)

Characteristics	Species Code		
	Mu	Nu	X1
CELLS			
Type	rod	rod	rod
Cell diameter, microns	0.5	0.9	0.5
Cell length, microns	1 - 2	3 - 4	1 - 2
Gram-staining Characteristics	negative	Gram-variable	negative
Spore-staining Characteristics	negative	central spores	negative
Aggregation	single, clusters	chains	clusters
CULTURES			
Oxygen requirements ¹	aerobe	aerobe	aerobe
Ethylene oxide resistance ²	-	+++	-
Environmental requirements ³			
TSA at 55°C	-	-	-
TSA at 37°C	++	+++	+++
TSA at 25°C	++	++	++
Sab at 25°C	+	++	+
AA at 37°C			
Population distribution in broth ³ :			
Uniform	--	yes	--
Sediment	--	yes	--
Clumps or filaments	--	no	--
Pellicle	--	yes	--
Location of Pellicle	--	top	--
Clear zone at top	--	no	--
Turbidity of culture in broth	--	dense	--
COLONIES			
Colony type on agar	smooth	rough	smooth
Opacity	translucent	opaque	transparent
Color	yellow	gray-white	light yellow
Diameter, millimeters	2	10	1/2
Edge	round	lobed	round
Texture	shiny	dull	shiny
Profile	convex	flat	convex

TABLE II (Continued)

Characteristics	Species Code		
	Omicron	Pi	Rho
CELLS			
Type	rod	rod	rod
Cell diameter, microns	0.5	0.9	0.5
Cell length, microns	1 - 2	3 - 4	1 - 2
Gram-staining		Gram-positive	
Characteristics	negative	Terminal Granules	negative
Spore-staining			
Characteristics	negative	central spores	negative
Aggregation	clusters	chains	clusters
CULTURES			
Oxygen requirements	aerobe	aerobe	aerobe
Ethylene oxide resistance ¹	-	-	-
Environmental requirements ²			
TSA at 55°C	-	-	-
TSA at 37°C	+++	+++	+++
TSA at 25°C	++	++	++
Sab at 25°C	+	+	+
AA at 37°C			
Populations distribution in broth ³ :			
Uniform	--	--	--
Sediment	--	--	--
Clumps or filaments	--	--	--
Pellicle	--	--	--
Location of pellicle	--	--	--
Clear zone at top	--	--	--
Turbidity of culture in broth	--	--	--
COLONIES			
Colony type on agar	smooth	rough	smooth
Opacity	transparent	opaque	transparent
Color	light yellow	gray-white	light yellow
Diameter, millimeters	1	7	1/2
Edge	round	lobed	round
Texture	shiny	dull	shiny
Profile	convex	flat	convex

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TABLE II (Continued)

Characteristics	Species Code		
	Sigma	Tau ⁵	Upsilon ⁵
CELLS			
Type	rod	coccus	coccus
Cell diameter, microns	0.5	0.6 - 0.8	0.8 - 1.0
Cell length, microns	1 - 2	--	--
Gram-staining			
Characteristics	negative	Gram-positive	Gram-positive
Spore-staining			
Characteristics	negative	--	--
Aggregation	clusters	diploid, tetrad, small irregular clusters	small irregular clusters
CULTURES			
Oxygen requirements	aerobe	facultative	facultative
Ethylene oxide resistance ¹	-	+	+++
Environmental requirements ²			
TSA at 55°C	-		
TSA at 37°C	+++		
TSA at 25°C	++		
Sab at 25°C	+		
AA at 37°C	-	+	+
Populations distribution in broth ³ :			
Uniform	--	no	no
Sediment	--	none	none
Clumps or filaments	--	filaments	no
Pellicle	--	--	yes
Location of pellicle	--	--	upper zone
Clear zone at top	--	yes	no
Turbidity of culture in broth	--	filaments	confined to upper zone
COLONIES			
Colony type of agar	smooth	smooth	smooth
Opacity	translucent	opaque	opaque
Color	yellow	white	white
Diameter, millimeters	1	1 - 2	1 - 2
Edge	round	round	round
Texture	shiny	shiny	shiny
Profile	convex	convex	convex

TABLE II (Continued)

Characteristics	Species Code		
	Phi ⁴	Chi ⁵	Psi ⁵
CELLS			
Type	rod	rod	rod
Cell diameter, microns	0.5 - 0.9	0.9	1 - 1.5
Cell length, microns	3 - 4	4 - 5	4.0
Gram-staining Characteristics	Gram-positive terminal granule	Gram-positive	Gram-positive
Spore-staining Characteristics	terminal spores	negative	negative
Aggregation	singles and chains	chains	chains
CULTURES			
Oxygen requirements	aerobe	facultative	facultative
Ethylene oxide resistance ¹	+	+++	+++
Environmental requirements ²			
TSA at 55°C			
TSA at 37°C	+++		
TSA at 25°C			
Sab at 25°C		+	+
AA at 37°C			
Populations distribution in broth ³ :			
Uniform	yes	no	no
Sediment	no	none	none
Clumps or filaments	no	no	no
Pellicle	yes	yes	yes
Location of pellicle	top	upper zone	upper zone
Clear zone at top	no	no	no
Turbidity of culture in broth	dense	confined to upper zone	confined to upper zone
COLONIES			
Colony type on agar	rough	mucoid	mucoid
Opacity	opaque	translucent	translucent
Color	light yellow	cream	cream
Diameter, millimeters	6 - 10	2 - 3	2 - 3
Edge	lobed-rhizoid	irregular	irregular
Texture	dull	membranous	membranous
Profile	flat	flat	flat

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TABLE II (Continued)

Characteristics	Species Code		
	Omega ⁵	Aleph ⁵	Beth ⁵
CELLS			
Type	coccus	coccus	coccus
Cell diameter, microns	0.8 - 1.0	0.6 - 0.8	0.8 - 1.0
Cell length, microns	--	--	--
Gram-staining	Gram-positive	Gram-positive	Gram-positive
Characteristics			
Spore-staining	--	--	negative
Characteristics			
Aggregation	diploid, tetrad, small clusters	single, diploid, triad and tetrad	single, diploid, triad, tetrad, small clusters
CULTURES			
Oxygen requirements	facultative	facultative	facultative
Ethylene oxide resistance ¹			++
Environmental requirements ²			
TSA at 55°C			
TSA at 37°C			
TSA at 25°C			
Sab at 25°C			
AA at 37°C	+	+	+
Populations distribution in broth ³ :			
Uniform	--	--	no
Sediment	--	--	none
Clumps or filaments	--	--	no
Pellicle	--	--	yes
Location of pellicle	--	--	upper zone
Clear zone at top	--	--	no
Turbidity of culture in broth	--	--	confined to upper zone
COLONIES			
Colony type on agar	smooth	smooth	smooth
Opacity	opaque	opaque	opaque
Color	white	white	white
Diameter, millimeters	1 - 2	1 - 2	1 - 2
Edge	round	round	round
Texture	shiny	shiny	shiny
Profile	convex	convex	convex

TABLE II (Continued)

Characteristics	Species Code		
	Gimel ⁵	Daleth ⁵	He ⁵
CELLS			
Type	rod	coccus	rod
Cell diameter, microns	0.9	1.0 - 1.5	0.9
Cell length, microns	4.0	--	4.0
Gram-staining Characteristics	Gram-positive	Gram-positive	Gram-positive
Spore-staining Characteristics	Central spore	--	negative
Aggregation	Single and chain	tetrad, small clusters	single, chain
CULTURES			
Oxygen requirements ¹	facultative	facultative	facultative
Ethylene oxide resistance ²	+++	+	++
Environmental requirements			
TSA at 55°C			
TSA at 37°C			
TSA at 25°C			
Sab at 25°C			
AA _e at 37°C	+	+	+
Populations distribution in broth ³ :			
Uniform	no	no	no
Sediment	none	none	none
Clumps or filaments	no	none	no
Pellicle	yes	yes	yes
Location of pellicle	upper zone	upper zone	upper zone
Clear zone at top	no	no	no
Turbidity of culture in broth	confined to upper zone	confined to upper zone	confined to upper zone
COLONIES			
Colony type on agar	mucoid	smooth	mucoid
Opacity	translucent	opaque	translucent
Color	cream	white	cream
Diameter, millimeters	2 - 3	1 - 2	2 - 3
Edge	irregular	round	irregular
Texture	membranous	shiny	membranous
Profile	flat	convex	flat

TABLE II (Continued)

Characteristics	Species Code		
	Cl. sporogenes ⁵	Zayin ⁵	Cheth
CELLS			
Type	rod	rod	mold spore
Cell diameter, microns	0.5	0.9	3 - 4
Cell length, microns	3.0	4.0	5 - 7
Gram-staining Characteristics	Gramm-positive	Gram-positive	
Spore-staining Characteristics	Terminal spore		
Aggregation	single, chain	single, chain	
CULTURES			
Oxygen requirements	anaerobe	facultative	aerobe
Ethylene oxide resistance ¹	+	+++	
Environmental requirements ²			
TSA at 55°C			
TSA at 37°C			
TSA at 25°C			
Sab at 25°C			++
AA at 37°C	+	++	
Populations distribution in broth ³ :			
Uniform	no	no	
Sediment	none	none	
Clumps or filaments	no	no	
Pellicle	yes	yes	
Location of pellicle	middle third	at the top	
Clear zone at top	yes	yes	
Turbidity of culture in broth		dense	
COLONIES			
Colony type on agar	mucoid	smooth	opaque
Opacity	translucent	transparent	green
Color	cream	light yellow	20
Diameter, millimeters	2 - 3	1	round
Edge	irregular	round	felt like
Texture	membranous	shiny	flat, concentric
Profile	flat	convex	rings

TABLE II (Continued)

Footnotes

1. Ethylene oxide resistance was measured by a screening test which determined whether or not the species could grow in fluid culture in the presence of ethylene oxide in aqueous solution. The ethylene oxide-screening broth was prepared by mixing into sterile broth a solution of one milliliter of cold liquid ethylene oxide in nine milliliters of cold water in the ratio 1 of solution to 25 of broth. Trypticase soy broth was used for all species except those identified by a superscript "5". For these, fluid thioglycollate medium was used. This screening test was based on a procedure developed in Task I which permitted comparison with the results obtained by other investigators.

The ethylene oxide resistance of species omega and alpha were not measured because only one colony on each of their respective initial identifying agar plates was sufficiently isolated that a mixed population could be avoided. The minus sign for ethylene-oxide resistance indicates that this species would not grow in the ethylene oxide-screening broth whereas the plus signs indicate that it did grow. The greater the number of plus signs the greater the vigor of the observed growth in the screening broth. One or more colonies identified in Table I were collected from the surface of the agar and transferred on a loop to the broth for incubation. No attempt was made to quantitate the infectivity of the species for ethylene oxide-screening broth.

2. The environmental requirements tests for aerobes were made by streaking the surface of the indicated agars with a loop of the inoculum taken from a colony identified in Table I. The population appeared as a continuous colony along the streak. A single plus sign indicates a visible colony, two plus signs indicate a moderately dense population, and three plus signs indicate very vigorous growth along

the streak. A blank indicates that no test was made.

The Trypticase soy agar (TSA) and the Sabourauds dextrose agar (Sab) were obtained from Hyland Laboratories as Pre-Med Monoplates. The anaerobic agar (AA), fluid thioglycollate medium (FTM), and Trypticase soy broth (TSB) were prepared from dry powder supplied by Baltimore Biological Laboratory.

The environmental requirements tests for the facultative species were made by observing colonies on the surface of anaerobic agar and in deep tubes of anaerobic agar.

3. The observations about the cultures of the several species were made on tubes containing the species in the ethylene-oxide screening cultures described in footnote No. 1 above. Some of the species were grown in fluid culture without ethylene oxide. The presence of ethylene-oxide did not change the appearance of the culture. In most cases, however, the cultures in FTM appeared quite different from cultures of the same species in TSB. The aerobes confined their growth to a band at the top of the broth but large amounts of cells collected at the interfaces between the aerobic and the anaerobic zones to form a structure resembling a pellicle. Below this interface, the cells formed a clumpy turbidity with varying amounts of clear broth. In TSB, the aerobes produced a more or less uniform turbidity. In every case population growth in FTM removed all shades of red color from the broth.
4. This species did not occur in the solid propellant but has occurred as a contaminate in our laboratory. It was present in large numbers in the dust specimens studied in an earlier program. It grows very rapidly and suppresses the growth of alpha when the two occur together. It has been identified as B. lichenformis sp.
5. The colonies of these organisms were isolated initially by incubation of an inoculum taken from the incubated baby food jars and placed on the surface of anaerobic agar in a Brewer anaerobic dish. They were incubated at 37°C.

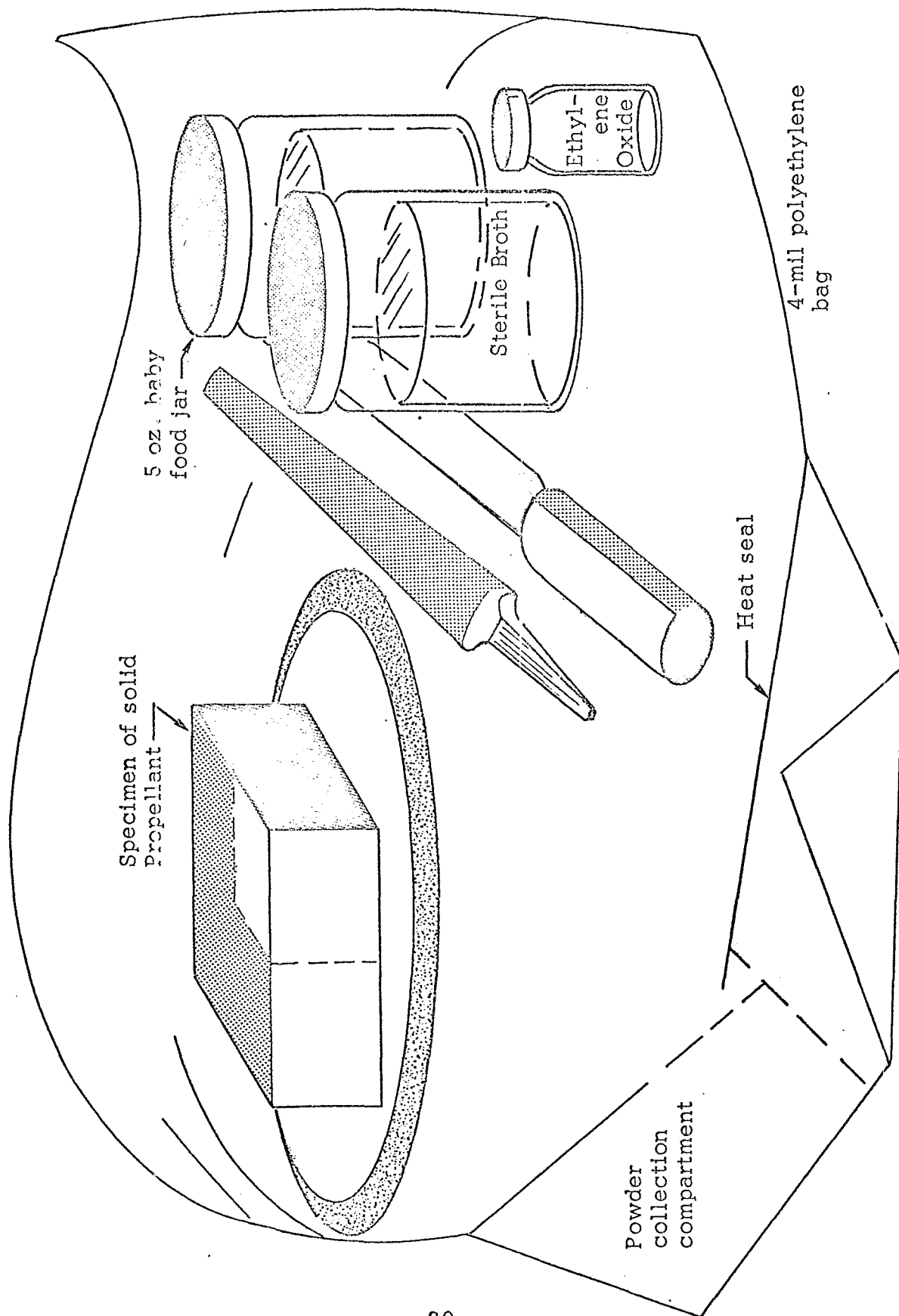


Figure 1. Isolator, Specimen, Tools, and Supplies for Sampling Solid Propellant Specimen

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MICROORGANISMS IN SOLID MATERIALS
TASK III:
RECOVERY LEVELS OF MICROBIAL ORGANISMS
INOCULATED INTO SOLID PROPELLANT SPECIMENS

(Mod. 1)

VOLUME V

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I. INTRODUCTION

The objective of Task III is to determine the minimum number of microorganisms that can be inoculated in solid propellant and subsequently detected by cultural techniques. This extinction value (minimum number of microorganisms allowing detection) defines the limit for detection of growth of the added inoculum. In Task IV experiments, selected levels of organisms will be added as inocula to solid propellants; sterilization procedures will be applied and the propellant will be subsequently cultured. The absence of growth of the added inoculum does not necessarily mean that no organisms survived the sterilization procedures. Rather, it indicates that fewer organisms survived than represented by the extinction value established in Task III. Obviously, the smaller the size of the inoculum that can be detected by growth in culture, the greater the degree of confidence one may place on the effectiveness of the sterilization procedures applied.

The methods for culturing the inoculated propellant was purposefully adapted for detecting the organism present in the inoculum. It must be emphasized that the conditions for assessing the presence of microorganisms in this task may not be ideally suited for the detection of all organisms.

During the course of work on this task it became apparent that certain modifications must be made in the recovery techniques used in earlier work in order to increase the sensitivity of detection of organisms. Certain inhibitory substances were present in the propellant that were not found in other solids that were studied and these inhibitors had to be neutralized before a maximum recovery efficiency could be obtained. In addition to the neutralization approach, improvements were made in the method of pulverizing the material and in the preparation of the media. All of these changes tend to optimize the results and as a result the efficiency of the recovery was substantially improved over the work done in previous studies.

The results of our work in Task III have also indicated that ultimately these basic recovery techniques can be refined and extended until they may ultimately reach an efficiency of less than one microorganism per cubic centimeter.

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II. METHODS

A. PREPARATION AND ASSAY OF INOCULUM

The organisms studied in Task III were Bacillus subtilis var. niger, Cl. sporogenes, Serratia marcescens, Ulocladium, and Staphylococcus epidermidis.

1. Bacillus subtilis and Serratia marcescens

These organisms were suspended in dilution vials and viability counts were performed following decimal dilution by plate counting techniques within three days of the time the samples were prepared for submission to JPL. The organisms were adjusted to yield final concentrations of 10^2 , 10^3 , 10^4 organisms per cm^3 propellant. The organisms were intimately intermixed with heat sterilized dry Celite (2-3 grams per each level of organism inoculum), and submitted to JPL for incorporation in propellant specimens.

2. Staphylococcus epidermidis

This organism was maintained on Trypticase Soy Agar (BBL). Inocula from these agar slants were made into Trypticase soy broth. During the period of accelerated growth (logarithmic phase) the organisms were recovered from the culture media by centrifugation. They were washed three times in sterile distilled water and resuspended in a small volume of sterile distilled water. The cells were assayed by decimal dilution and plating techniques utilizing Trypticase Soy Agar.

3. Clostridium sporogenes

The Clostridial spores were prepared by growing vegetative organisms in Brain Heart Infusion Broth (DIFCO). Calcium chloride was added as a supplement to the media at a final concentration of 1% to enhance sporulation. The organisms were harvested by centrifugation at approximately $1000 \times g$

and the sediment was examined microscopically to insure that they were in the form of spores. The supernatant fluid was discarded and the spores were washed three times by alternately centrifuging and resuspending in sterile distilled water. The washed resuspended cells were subjected to exposure to heat at 80°C for a period of 10 minutes to destroy surviving vegetative forms and to ensure that only the spores survive. Following this heat shock step, the cells were stored at 5°C until required as an inoculum. In order to provide the inoculum at specific levels for JPL, the cells were assayed by decimal dilution in anaerobic deep agar tubes and by the use of Brewer Anaerobic Agar (BBL) plates.

4. Ulocladium

The Ulocladium was grown on Sabouraud agar medium (DIFCO). The organisms were harvested at the end of the first week of growth in distilled water and were subjected to sonication in an Acoustica Associates Model DR 50 AH ultrasonic bath for a period of 20 minutes at 40 to 60 ma. at a power output which produced cavitation. Following sonication the cells were centrifuged at 1000 x g for 10 minutes, the supernatant fluid discarded and resuspended in sterile distilled water. This procedure was repeated twice. The cells were then resuspended in sterile distilled water in a sterile solution bottle and maintained at 5°C until required for use as inoculum. At the time of submission of the organism to JPL, plate counts on Sabauroud agar medium (DIFCO) were performed.

In all instances the assayed microorganisms were mixed in small volume with a proportionately greater volume of heat sterilized Celite (Johns-Manville). The total weight of the Celite used for each level of organism inoculum ranged between 2 and 3 grams. With the exception of the "mixed inoculum" all organisms were supplied to yield three levels of concentration 10^2 , 10^3 , $10^4/\text{cm}^3$ propellant. The mixed inoculum was supplied at levels to yield 10^1 , 10^2 , and 10^3 organism/ cm^3 propellant. The contents of the vials were added to 1000 grams of propellant formula by personnel at JPL.

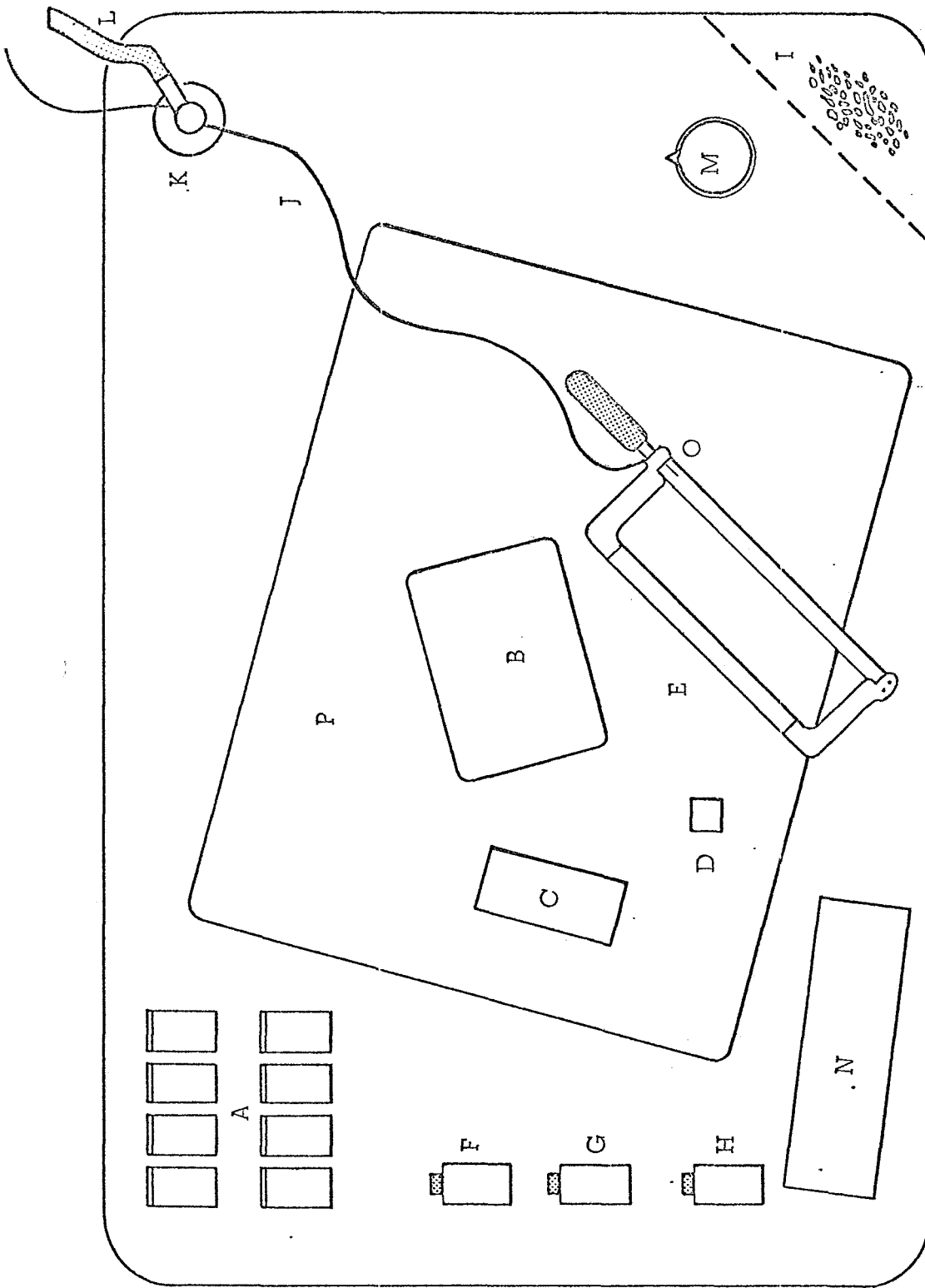
The propellant is composed of:

- 1) ammonium perchlorate
- 2) alroperse (reaction product of equal mixture of stearic acid and diethanolamine)
- 3) phenyl-beta-naphthylamine
- 4) 1, 1, 1-trimethylol propane
- 5) ferric acetylacetonate
- 6) 2, 4-toluene diisocyanate
- 7) poly propylene glycol
- 8) aluminum oxide

The propellant was returned to Dynamic Science as soon as possible following a 3 to 4 day curing period and immediately assayed for surviving organisms.

B. PULVERIZATION OF PROPELLANT

The propellant was enclosed in polyethylene sterilant bags (to liter capacity) as shown in Figure 1. These bags were equipped with a Teflon gland which permits the maintenance of a gas-tight system during the time period in which the contents of the bag are being sterilized. Each sterilant bag contained 60 to 70 ml of ethylene oxide (Matheson research grade), in a shallow dish. Several paper towels previously moistened with water were included in the bag to assure that humidity was near 50 percent. The effectiveness of ethylene oxide sterilization is markedly increased with elevated humidity. This atmosphere was maintained within the bag for a period of 12 to 16 hours to insure ample time for surface sterilization. The gland attachment, following sterilization, is subsequently attached to autoclave sterilized cotton packed exhaust tubing. The bag is evacuated using a water vacuum pump, after which the tubing is detached and sterile air is allowed to refill the bag and the bag is re-evacuated. Alternate exhaustion of the ethylene oxide from the bag and replacement with sterile air is thus accomplished. This procedure was repeated three times. Several dry paper



LEGEND: A = Sterile water & culture media F = 0.1 N NaOH K = Gland
 B = Wooden block G = 0.1 N HCl L = Bacteriological cotton utilized
 C = Propellant H = Cysteine M = Beaker cont. ethylene oxide
 D = 5 ml beaker I = Waste Cuttings N = Paper towels (12)
 E = Saw J = Copper wire to ground O = Thermistor

Figure 1. Sterile Polyethylene Enclosure

towels were included in the sterilant bag to remove droplets of moisture from the internal surface of the bag. A segment of copper wire approximately 0.5 mm in diameter was attached through the conductive portion of the gland to a suitable ground. On the inner aspect of the bag the copper wire was attached to the blade of the saw. These precautions were taken to insure that static electrical discharge could not take place within the bag. Such discharge would be improbable since the humidity of the bag was maintained at or near 40 percent.

Previous studies on methods for pulverizing solid propellant resulted in limited and variable recoveries of organisms. The following techniques were evaluated in previous studies on solid propellant and found to be relatively ineffectual either in providing sufficient pulverization or in adversely affecting the organisms. These included the following techniques:

- 1) thin slicing with a blade-type microtome
- 2) drilling specimen propellant while submerged in culture media
- 3) abrasion with a "stickle back" rasp
- 4) abrasion with various types of files
- 5) homogenization in a Waring blender-type homogenizer

The use of saws of various conformations showed advantages over all previously attempted methods of pulverization. The blade found most serviceable was determined to contain 18 teeth per inch*. Several manufacturers provide such hacksaw blades. Some, however, contain toxic coatings which interfere with growth of microorganisms. A thermistor was attached to either the front one inch or the back one inch of the blade and a slow rate of sawing was used. The rate of sawing was so regulated that an increase in temperature of not more than 1°C resulted. Because the temperature at the saw tooth propellant interface may be several degrees higher than that indicated by the thermistor, the rate of sawing was adjusted to minimize possible thermal inactivation of organisms.

*Blade No. 1218-3, Clemson Bros., Inc.

The propellant was removed from the plastic container prior to placing it in the sterilant bag in order to facilitate surface sterilization of the propellant. Following the previously described sterilization step and the subsequent removal of the ethylene oxide from the bag, the propellant was scored to a depth of 2-4 mm. These cuttings were moved to a corner of the bag and sealed off. Previous tests had indicated that the outermost cuttings of the propellant were inhibitory to the growth of test organisms. The sampling areas of the propellant are shown in Figure 2. An attempt was made to sample at a random depth including sites near and distant from the surface. Roughly equivalent quantities were sampled from each of the indicated areas.

Beta propiolactone in place of ethylene oxide was used in 100 percent humidity for a 3-hour exposure period to induce surface sterilization. Our experience thus far indicates it offers the following advantages:

- 1) There is decreased permeation of sterilant through the interstices of the propellant.
- 2) Due to an approximate 20 fold increase in kill rate over ethylene oxide, shorter time periods are required to achieve surface sterilization. This also minimizes diffusion below the surface of the propellant.
- 3) The tendency for particles to agglomerate is decreased.

The particle distribution of the sawed propellant is indicated in Figure 3. Microscopically in all sizes of particles studied, innumerable fine cracks and checks were noted which likely have continuity with the interstices of even the largest particles. The larger particles appeared to result by fusion of smaller particles. This fusion effect could not be reversed by treatment with the Waring blender, sonication, or by treatment with wetting agents. Although Tween 80* was included in the culture media and did

*polyoxyethylene-sorbitan-monoaleate (Atlas Powder Co.)

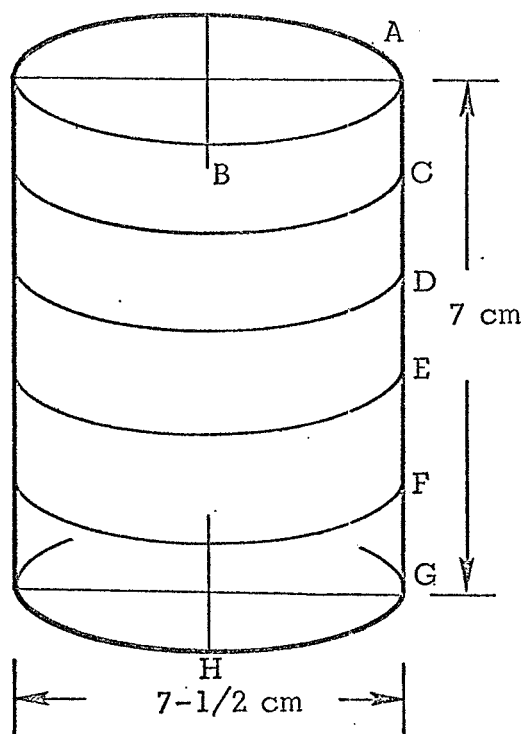


Figure 2. Sampling Areas Used For Solid Propellant

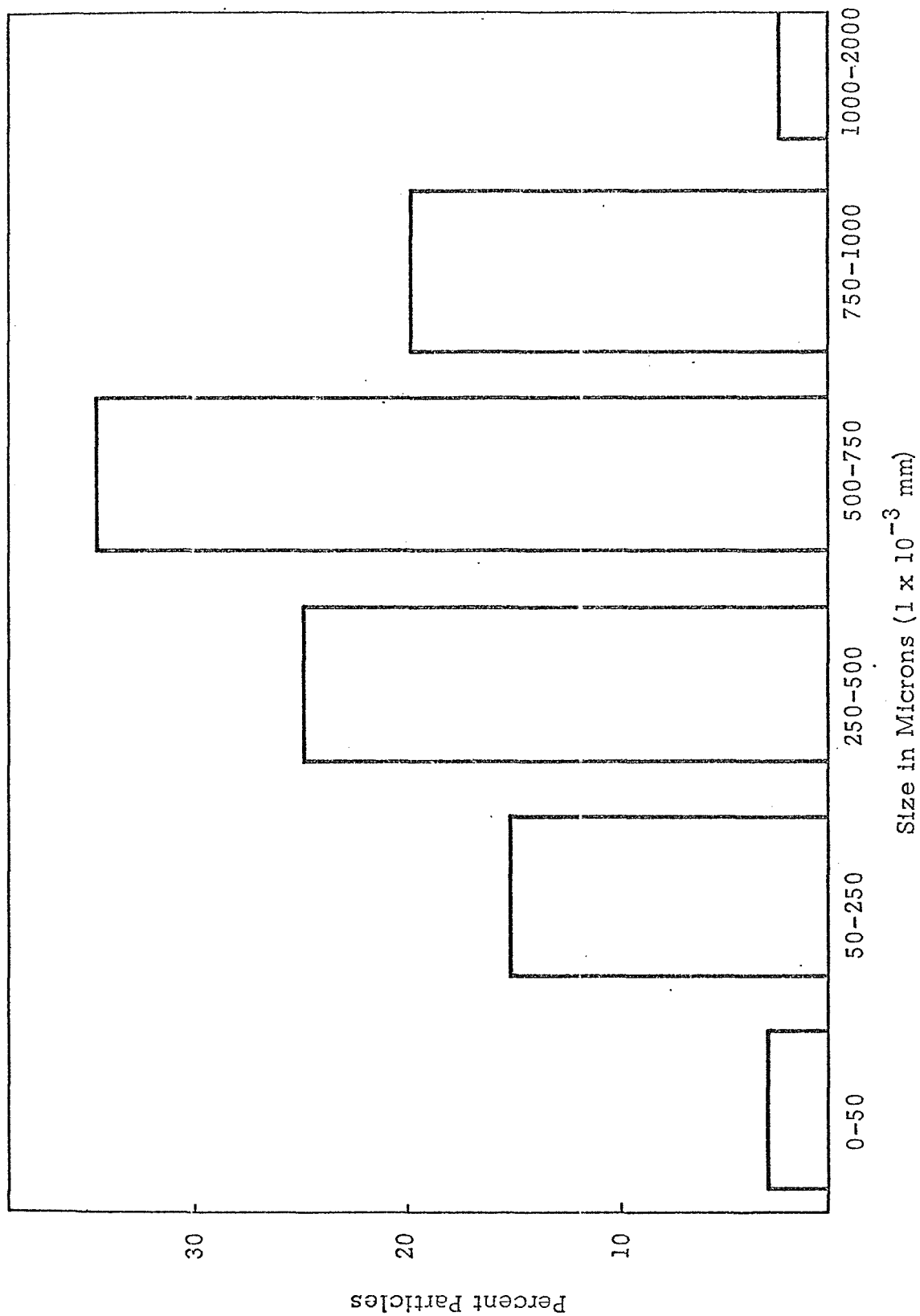


Figure 3. Size Distribution of Pulverized Propellant

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provide superior recoveries (Figure 4), it does not appear that such recoveries were the result of inhibition of particle agglomeration.

The propellant particles obtained by sawing were placed in a 5 ml beaker. The beaker, when completely filled, contains 3.2 grams \pm 200 mg. The quantities of propellant used in the culture studies consisted of multiples of 3.2 grams.

C. LEACHING OF PROPELLANT

The propellant while in the sterilant bag was added to a screw cap bottle containing 120 ml of distilled sterile water and a sterile Teflon coated magnetic stirring bar. The sterile screw cap containers were placed on a magnetic stirrer for 15 minutes. The supernatant fluid was treated as indicated in Figure 5. The redox potential of the leached propellant and the supernatant fluid was measured and this potential was adjusted to that considered optimum for each microorganism cultured. The pH was adjusted to 7.2 - 7.4 using sterile 1/10 normal acid or base except in the case of Ulocladium the pH was adjusted to 4.0 - 4.5.

The supernatant fluid was divided into three portions while in the sterile enclosure. One portion was centrifuged, stained with fluorescent dye, and examined microscopically for the presence of organisms. The second and third portions were added to culture media containing 1% Tween 80. The final volume of the leached supernatant fluid and culture medium was established by prior model experiments considering the inhibitory effect of the perchlorate from the propellant on the growth rate of the microorganism studied.

The ratio by weight of propellant to sterile distilled water which provided optimal recovery was determined to be 1:10. In previous studies, only the ability of a large inoculum of organisms to grow in the presence of the various propellant ingredients was determined. In the present study it was observed that concentrations of ammonium perchlorate as low as 0.5% interfered with the growth of some of the organisms.

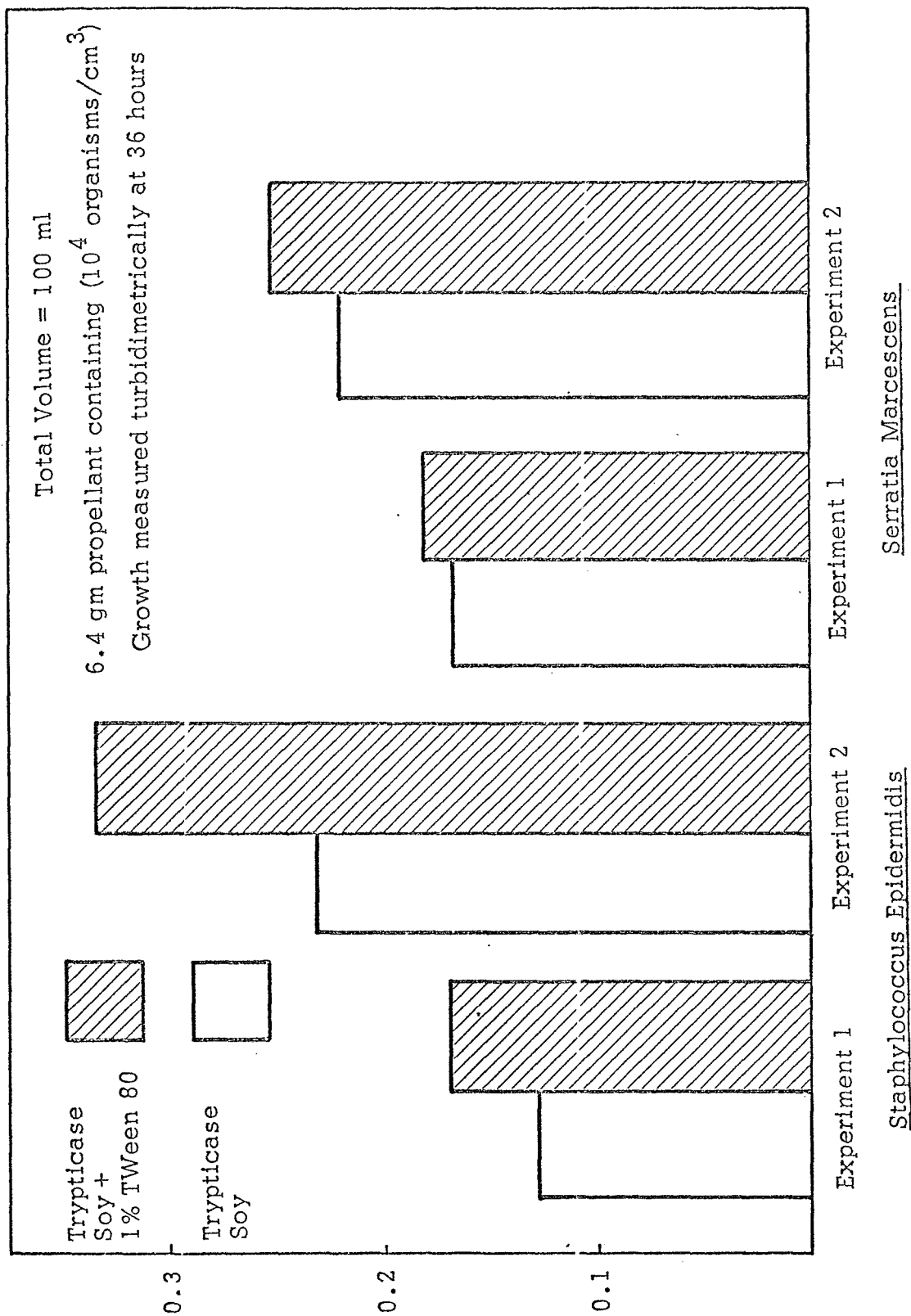


Figure 4. Effect of Tween 80 on Growth of Selected Bacteria in Propellant as Determined Turbidimetrically

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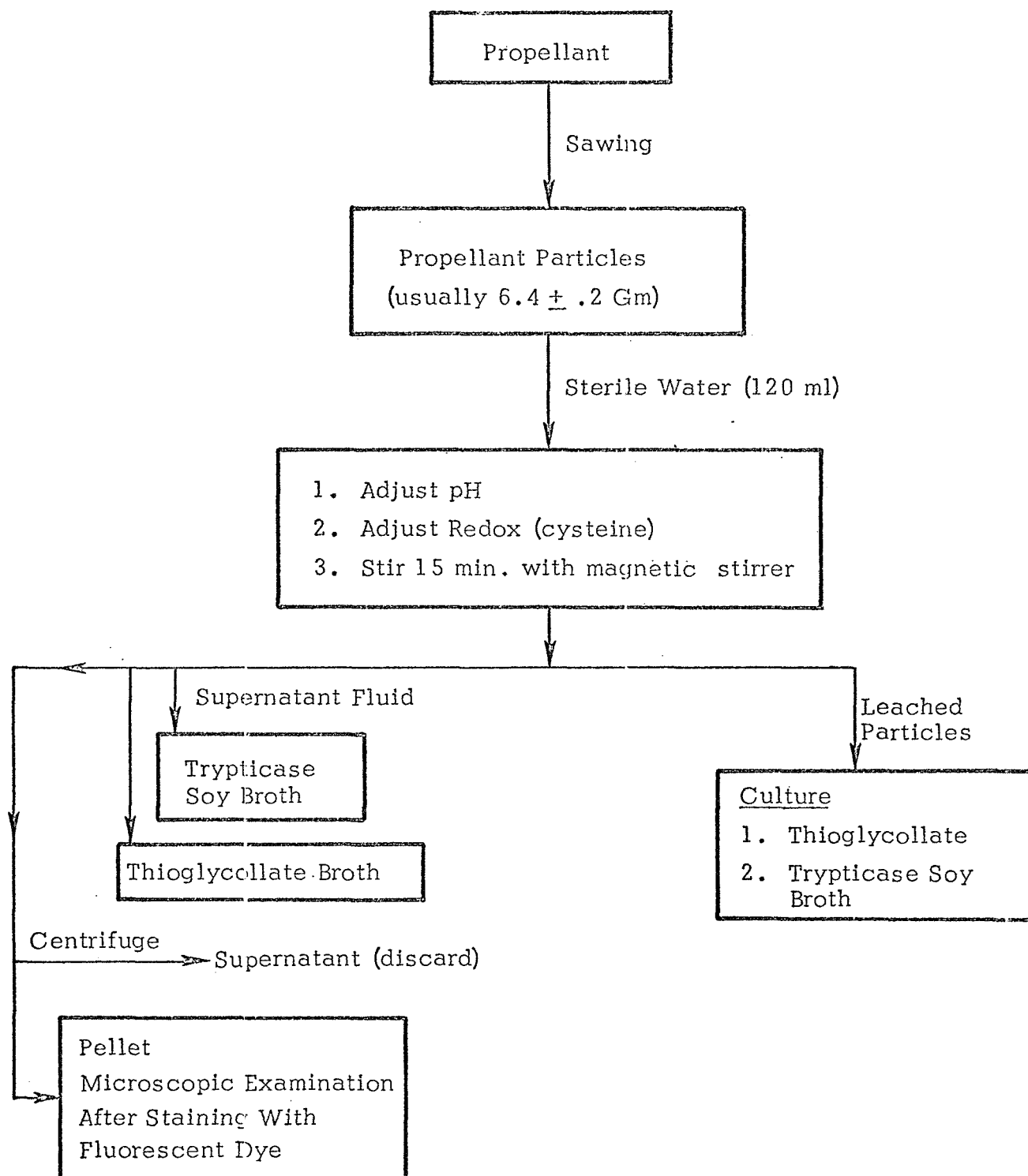


Figure 5. Summary Chart of Pulverization and Culture Methods

To the vessel containing the leached propellant an aliquot of cysteine was added to reduce the redox potential to the desired level. This level was previously arrived at by model experiments utilizing a sample of propellant outside of the bag. This provided a method for adding the required amount of reductant to the vessel while in a sterile environment.

In all cultural procedures routine controls were included for sterility of the enclosure's environment, the sterility of the growth media, and to insure the capability of the growth media to support the growth of low levels of microorganisms. Usually the latter control contained 10 and/or 10^2 levels of organisms. The temperature of incubation was maintained at 37°C . Additional cultures were maintained at 25°C for Ulocladium.

III. EXPERIMENTAL RESULTS

Recovery of test organisms was successful at 10^2 level of inoculum in all cases (Tables 1 - 6). B. subtilis, Cl. sporogenes and Staph epidermidis were recovered at 10^1 in the mixed inoculum (Table 6). Several contaminating types of organisms were recovered as well as the organism used in the inoculum. The time required for any particular culture to show evidence of bacterial or fungal growth varied considerably. The cultures containing organisms at the 10^4 level consistently showed evidence of growth prior to growth appearing in 10^3 , and growth was observed at 10^3 prior to 10^2 .

The organisms were identified by classical bacteriologic methods in order to verify that the growth observed in the culture vessels was due to the inoculum. In some instances growth due to the presence of contaminants in the propellant grew more rapidly than the inoculum. This was particularly true in some of the culture vessels containing the lowest level of inoculum. In some instances this made more difficult the task of recovery, and in some cases it may have obscured the recovery of the added microorganism.

Selected media and selective conditions were used in some experiments to inhibit the growth of contaminating organisms. These techniques were particularly useful when attempting to isolate Ulocladium. Although this fungal organism grows at a more rapid rate than many contaminating fungi, it does not grow as rapidly as many strains of microorganisms. By reducing the pH of the growth medium to 4.0 - 4.5 and utilizing a low ionic strength medium, profuse growth of Ulocladium was consistently observed under conditions which strongly inhibited the growth of contaminating bacteria.

Recovery of vegetative forms of Cl. sporogenes was poor in instances in which no attempt was made to counteract the strong oxidants present in the propellant. Following the addition of an optimal quantity of reducing agent and adjustment of the growth medium to the ideal redox potential

recovery of this organism was generally superior to recovery of any of the other inoculum tested.

The leached supernatant fluid consistently contained not only microorganisms used in the inoculum, but also contained variable quantities of contaminating microorganisms. Preliminary experiments have been carried out to determine the viability of these recovered organisms using other than cultural methods. Tetrazolium compounds in the presence of metabolically utilizable organic substrates lead in some instances to the appearance of reduced dye on the outer surface of microorganisms. Some of these organisms did not reduce the dye and therefore were likely either not viable or did not contain the enzymes which could be detected by the use of this dye. These experiments are at an early stage of study and do not represent a finalized experimental method.

TABLE 1

RECOVERY LEVEL OF INOCULATED PROPELLANT

Organism: B. subtilis var. niger

	Average time (days) required for detectable growth			Sterility Control	Comments
	Inoculum Level/cm ³	10 ²	10 ³	10 ⁴	
<u>Leached propellant particles</u>					
Trypticase soy broth	8I 4C	4I	2I	-	<u>Contaminants:</u> gm. + pos. cocci, gm. + rod central spore
Fluid thloglycollate	5I 2C	3I 4C	3I	-	
<u>Supernatant water extract</u>					
Trypticase soy broth	11I	7I 6C	4I	-	<u>Contaminants:</u> gm. + rod with granules, gram + rod with central spore, unidentified fungus
Fluid thloglycollate	12I 10C	7I	4I	-	
<u>Bacteriostasis control</u>					
Trypticase soy broth	4I	-	-	-	
Fluid thloglycollate	3I				
<u>Sterility control</u>					
Trypticase soy broth				0/4	
Fluid thloglycollate				0/4	

I = inoculum
 C = contaminant
 0 = no detectable growth

TABLE 2

RECOVERY LEVEL OF INOCULATED PROPELLANT

Organism: Cl. sporogenes

	Average time (days) required for detectable growth			Sterility Control	Comments
	Inoculum Level/cm ²	10 ³	Propellant 10 ⁴		
<u>Leached propellant particles</u>					
Anaerobic broth	4I 6C	3I	2I		<u>Contaminants:</u> gm. + rods with central spore small gram + rods without spores
Fluid thioglycollate	5I	5I 2C	5I		
<u>Supernatant water extract</u>					
Anaerobic broth	6I	5I	3I		Contaminants: gm. + rods with central spore
Fluid thioglycollate	0 3C	10I	2I		
<u>Bacteriostasis control</u>					
Anaerobic broth	2I				
Fluid thioglycollate	3I				
<u>Sterility control</u>					
Anaerobic broth				0/4	
Fluid thioglycollate				0/4	

I = inoculum
 C = contaminant
 0 = no detectable growth

TABLE 3

RECOVERY LEVEL OF INOCULATED PROPELLANT

Organism: Staphylococcus epidermidis (α)

	Average time (days) required for detectable growth			Sterility Control	Comments
	10 ²	10 ³	10 ⁴		
<u>Leached propellant particles</u>					
Trypticase soy broth	111	51	21		<u>Contaminants: gm. --</u> <u>rod, short gm. + rod</u>
Fluid thioglycollate	81 6C	41 2C	11		
<u>Supernatant water extract</u>					
Trypticase soy broth	91	41	21		<u>Contaminants: gm. +</u> <u>rod with central spore</u>
Fluid thioglycollate	0	51 2C	31		
<u>Bacteriostasis control</u>					
Trypticase soy broth	41				
Fluid thioglycollate	41				
<u>Sterility control</u>					
Trypticase soy broth				0/4	
Fluid thioglycollate				0/4	

I = inoculum
 C = contaminant
 0 = no detectable growth

TABLE 4

RECOVERY LEVEL OF INOCULATED PROPELLANT

Organism: Serratia marcescens

	Average time (days) required for detectable growth			Sterility Control	Comments
	10^2	10^3	10^4		
<u>Leached propellant particles</u>					
Trypticase soy broth	10I	5I 3C	1I		<u>Contaminants:</u> large gm pos. rods, gm pos. cocci
Fluid thioglycollate	6I 1C	4I 6C	3I		
<u>Supernatant water extract</u>					
Trypticase soy broth	7I 10C	6I	3I		Contaminants: gm + rods with central spores, gm negative rods
Fluid thioglycollate	0	6I 3C	2I		
<u>Bacteriostasis control</u>					
Trypticase soy broth	2I				
Fluid thioglycollate	2I				
<u>Sterility control</u>					
Trypticase soy broth				0/4	
Fluid thioglycollate				0/4	

I = inoculum

C = contaminant

0 = no detectable growth

TABLE 5

RECOVERY LEVEL OF INOCULATED PROPELLANT

Organism: Ulocladium

	Average time (days) required for detectable growth			Sterility control	Comments
	Inoculum Level/cm ³	10 ²	10 ³	10 ⁴	
<u>Leached Propellant particles</u>					
Trypticase soy broth	9I 5C	6I	3I		<u>Contaminant: mold not Ulocladium</u>
Sabouraud broth	12I	5I 10C	5I		
<u>Supernatant water extract</u>					
Trypticase soy broth	0 7C		4I		<u>Contaminant: gm pos. rod central spore</u>
Sabouraud broth	16I		6I		
<u>Bacteriostasis control</u>					
Trypticase soy broth	3	-	-		
Sabouraud broth	4	-	-		
<u>Sterility control</u>					
Trypticase soy broth				0/4	
Sabouraud broth				0/4	

I = inoculum
 C = contaminant
 0 = no detectable growth

TABLE 6

RECOVERY LEVEL OF INOCULATED PROPELLANT

Mixed Inoculum Consisting of:

B = Bacillus subtilis, Ser = Serratia marcescens, U = UlocladiumCs = Cl. sporogenes, S = Staphylococcus epidermidis

	Average Time (days) required for detectable growth			Sterility Control
	10 ¹	10 ²	10 ³	
<u>Leached propellant particles</u>				
Trypticase soy broth	B10, S11	B8, Ser 10, S9	B4, Ser 6, S5	
Fluid thloglycollate	Cs12	Cs6, B10, Ser9, S9 U11, B14	B5, Cs3, Ser 5, S10 U7, B16	
Sarbouraud broth (pH 4-4, 5)				
<u>Supernatant water extract</u>				
Trypticase soy broth	B12, S14	B7, Ser 12, S9	B3, Ser 7, S 5, U 17	
Fluid thloglycollate	Cs11	Cs9, B 17, Ser 12 U 15	Cs2, B7, Ser6, U14 U10, B9, S16	
Sarbouraud broth (pH 4-4, 5)				
<u>Bacteriostasis control</u>				
Trypticase soy broth	B5, Ser4, S8, U12			
Fluid thloglycollate	Cs8, B6, S12, Ser14			
Sarbouraud broth (pH 4-4, 5)	U10			
<u>Sterility control</u>				
Trypticase soy broth				0/4
Fluid thloglycollate				0/4

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IV. DISCUSSION

Low levels of microorganisms $10^2 - 10^4/\text{cm}^3$ intentionally inoculated into solid propellants may be successfully detected by culture methods. The pulverization of the propellant by sawing has proven most useful in our studies. The pulverized samples obtained using this technique consistently give superior results to those obtained previously using other methods. Intermittent stirring with a magnetic stirrer aided the maintenance of aeration and also likely exposes new surfaces containing organisms.

Inhibitory substances were present in the propellant which interfered with the growth of all strains of microorganisms tested. It was essential that these inhibitory substances be removed from the solid propellant particles by leaching. One of the inhibitors present in dilute form in the leached fluid is ammonium perchlorate. The perchlorate may be reduced chemically by treatment with dilute strong reducing agents. The use of such agents had led to better recovery of microorganisms from both the leaching fluid and the pulverized propellant. The control of the reduction-oxidation potential of the growth medium may be as important as control of pH in the recovery of small numbers of microorganisms using culturing methods. Other inhibitory or potentially inhibitory substances include phenyl-beta naphthylamine, 1, 1, 1-trimethyl propane, ferric acetylacetonate, and 2, 4-toluene diisocyanate which are all constituents of solid propellants. In the present study there were no attempts to discern the order or magnitude of inhibition inducible by these substances, however, it is obvious that such experiments should be performed.

The use of fluorescent dyes in detecting the presence of organisms in the leached supernatant shows promise. This method is somewhat deficient in that there is no means of determining viability of organisms visualized with this technique.

Preliminary experiments with tetrazolium compounds and oxidizable organic substrates have proven promising. It has been possible to detect the presence of reduced dye on the surface of organisms leached from propellant and it is probable that this method will be extremely useful in propellant sterilization studies.

In a large percentage of the samples of propellant studied we have isolated microorganisms which were not present in the inoculum and which apparently represent JPL environmental contamination. This is to be expected since in the course of Task III investigations, no attempt has been made to provide the propellant in a state of sterility at the time the inoculum was incorporated into it.

V. SUMMARY AND RECOMMENDATIONS

In the course of the studies conducted in Task III of this program, we have detected microorganisms in inoculated solid propellant at all levels studied (10^2 , 10^3 , 10^4) and in some instances at $10^1/\text{cm}^3$. The presence of contaminating microorganisms in a high percentage of the propellant samples indicates that as future studies progress, preliminary steps may have to be taken to reduce the population of non-inoculated organisms. We have found that through the use of an improved means of pulverizing the propellant, control of pH, neutralization of toxic substances, and adjustment of redox potential it has been possible to materially improve detection of small numbers of organisms by culture. There are indicated areas of further improving the efficiency of culturing techniques for recovery of organisms from propellant. These include a more detailed study of the effects of certain constituents of the propellant on the test microorganisms, determination of optimum pH, and optimum conditions of electrostatic repulsion between propellant fragments and microorganisms.

The addition of neutralizers to growth media should be investigated. These neutralizers may reduce or reverse the effect of the constituents of the propellant on the microorganisms. This approach has been found to be useful in studies on the recovery of microorganisms previously exposed to hexachlorophene, organo-metallic compounds and quaternary ammonia compounds. Such neutralizers to counteract constituents of propellant may be incorporated into the growth media.

It is also possible that an excess of one or more growth factors may be required for the recovery of microorganisms inhibited by propellant constituents. The effect of an excess of such growth factors on growth should be systematically investigated. Two additional factors are important in the recovery of organisms from propellant. These include 1) the incorporation of high buffer capacity for organisms which produce organic acid and whose growth is inhibited by these acids, and 2) media of low ionic strength for the detection of contaminating organisms which are adversely affected by ionic strength.

It is apparent that with optimization of cultural techniques and the supplementation of other ancillary techniques that recoveries may be extended to less than 1 organism/cm³. The effects of the solid on the microorganism and/or the effects of pulverizing the solid material may preclude isolation of viable microorganisms by cultural methods. However, the enzymic activities of potentially viable organisms could provide a sensitive means of showing the presence of organisms which may still be in the living state. The physical and chemical conditions which induce sterility usually will destroy enzymic systems within the cell. For example, treatment with heat in sufficient quantity to cause sterilization will also cause denaturation of the protein moiety of the enzymes. Similarly, heavy metals, formaldehyde, phenol and similar agents cause concurrent sterilization and enzyme destruction.

One broad category of enzymic reactions which may be sufficiently sensitive include those which cause reduction of tetrazolium. The various substituted tetrazolium salts are characterized by water solubility and lack of visible color in the oxidized state and lack of water solubility and the development of intense color in the reduced state. Depending upon the nature of the substituents on the tetrazolium the color of the reduced dye may range from red to blue. Because of the change in phase of the reduced dye, it is possible to concentrate the dye following a prolonged exposure to the material containing organisms or organism fragments. An immiscible solvent could be added to the aqueous phase in small quantity to dissolve the reduced dye and at the same time concentrate it for photometric measurement if desired. Alternatively it would be possible with the light microscope to examine the particles produced by pulverization of the solid after incubation with the oxidizable organic substrate and the tetrazolium. In this case, the organisms and the organism fragments would be characteristically stained whereas the devitalized or nonviable organisms lacking the enzymic activities would not be stained by the dye.

Other enzyme assays which may be utilized for the detection of potentially viable organisms include cytochrome oxidase (using para-phenylene diamine which is converted to an intense purple color in the reduced state), peroxidase, and the reduction of NAD (nicotinamide adenine dinucleotide). The latter substances have a characteristic spectrum in the reduced state in the ultraviolet, but can also be measured more sensitively with fluorescence.

Solids can be treated with a fluorescent stain which will adhere strongly to all cell walls of microorganisms and may be so selected to fluoresce in a different region of the spectrum from the fluorescence (when present) characteristic of the solid itself. Some of this work has been reported in this task. Fragments of all cell walls of microorganisms will show intensely and can be easily detected by scanning methods using the fluorescence microscope. The principal areas that will require study include the suppression of interfering auto-fluorescent substances in certain solids and possibly the requirement for removing or destroying quenching substances in the mixture.